REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jul 1999 - 30 Jun 2002)

4. TITLE AND SUBTITLE
Improving Retroviral Vectors for Gene Therapy of Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9377

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This project aims to increase the transduction efficiency and target cell-specificity of retroviral vectors, thereby improving gene transfer to breast cancer cells. We have developed a novel replication-competent retroviral vector system for gene transfer to solid tumors which is highly efficient, as each tumor cell which is successfully transduced becomes itself a virus-producing cell and initiates further infection events even after the initial injection. Through this project, we have tested a number of strategies for targeting these replicating vectors specifically and exclusively to tumor cells, including (1) engineering a single-chain antibody directed against HER-2 into the viral envelope sequence, and (2) insertion of an immunoglobulin-binding domain into the envelope to allow modular conjugation of monoclonal antibodies. While we have successfully achieved incorporation of these modified envelopes into RCR virions and obtained some functional targeting in artificial target cells, we encountered technical difficulties in application of these strategies for targeting true human breast cancer cells. However, we have found that even untargeted RCR vectors exhibit highly tumor-selective replication in breast cancer xenograft models *in vivo*, and the efficient intratumoral transmission of suicide genes via replicative spread of the RCR vector results in significant inhibition of tumor growth upon pro-drug administration.

14. SUBJECT TERMS
Breast cancer, gene therapy, retroviral vectors, replication-competent retrovirus, targeting

15. NUMBER OF PAGES
56

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-99-1-9377

TITLE: Improving Retroviral Vectors for Gene Therapy of Breast Cancer

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REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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N. Fujihara July 1, 2002

PI - Signature **Date**

Table of Contents

Attachments (publications):

1. Logg, C. R., Logg, A. , Tai, C. K., Cannon, P. M., Kasahara, N. 2001. Genomic stability and natural selection of murine leukemia viruses containing insertions at the *env*-3' UTR boundary. *J Virol*, 75: 6989-6998.
2. Logg, C. R., Logg, A., Tai, C. K., Anderson, W. F., Kasahara, N. 2001. A uniquely stable replication-competent retrovirus vector achieves efficient gene delivery *in vitro* and in solid tumors *in vivo*. *Hum Gene Ther*, 12: 921-932.
3. Tai, C. K., Logg, C. R., Park, J. M., Anderson, W. F., Press, M. F., Kasahara, N. 2003. Antibody-mediated targeting of replication-competent retroviral vectors. *Hum Gene Ther*, 14: 789-802.

Final Report **July 1, 2002**

I. INTRODUCTION

Currently, one of the foremost obstacles to the practical implementation of gene therapy is the low transduction efficiency of gene delivery vectors *in vivo*. This project aims to improve gene transfer to breast cancer cells through the development of replication-competent retrovirus (RCR) vectors that are specifically targeted to human breast cancer cells. We have devised a novel construct design to produce replicating retrovirus vectors that have proven to be stable over multiple serial passages in cell culture, and that is capable of highly efficient gene delivery to breast cancer cells *in vivo* in a solid tumor xenograft model in nude mice. In this project, our aims were to construct replication-competent retrovirus vectors that are designed to specifically target breast cancer cells over-expressing HER-2, by the incorporation of targeting moieties such as peptide hormone or single-chain antibodies into the viral envelope, and to test whether such envelope modifications will now enable us to control the spread of these replication-competent virus vectors, allowing more efficient transduction of tumor cells while minimizing the risk to normal cells.

Through the course of this project, we have discovered that re-directing the binding tropism of the retroviral envelope is possible, but the increased binding specificity is achieved at the cost of a drastic decrease in overall infection efficiency, presumably because viral envelope modifications often result in inefficient coupling of the cell surface binding and virus-cell membrane fusion steps during the entry process. Although we tested different strategies for evolving retrovirus quasispecies that might be better adapted for efficient entry using the modified envelopes, we were not able to recover such species with drastic envelope modifications and observed reversion to wild type envelope with less extensive modifications. However, we also found that even without substantial envelope modification, the intrinsic inability of RCR vectors to enter quiescent non-dividing cells by itself results in highly tumor-selective replication *in vivo*, and in animal models we do not detect any extratumoral spread of the replicating retrovirus even using sensitive PCR methods. Such tumor-selectively replicating retrovirus vectors can achieve efficient transfer of marker genes and suicide genes throughout the entire tumor, and with pro-drug administration can achieve significant inhibition of tumor growth *in vivo*.

II. BODY

During the award period from July 1, 1999 to June 30, 2002, we have substantially accomplished the Tasks listed in the approved Statement of Work, shown below.

Statement of Work:

Task 1. Construction of RCR vectors targeted to human breast cancer cells (Months 1–8)
Task 2. Creation of RCR vector-producing cell lines (Months 8–14)
Task 3. Testing the tissue specificity of the virus in culture (Months 14–21)
Task 4. Transduction of human breast cancer xenografts *in vivo* (Months 21–36)

Research accomplishments:

***Task 1.* Construction of RCR vectors targeted to human breast cancer cells (Months 1–8)**

We applied the strategy of targeting specific cell types via envelope modification to replication-competent retrovirus (RCR) vectors, for gene therapy of breast cancer. Directing RCR vectors specifically to breast cancer cells provides a way to limit the spread of virus while enabling more efficient transduction of tumors.

Results:

We have constructed several RCR vectors targeted to breast tumor cells using two distinct types of modification to the envelope protein.

In order to obtain RCR vectors targeted to breast tumor cells, we constructed vectors containing modifications in the envelope gene that would allow specific binding of vector particles to proteins expressed on the surface of breast tumor cells. We have used two distinct approaches in targeting the vectors.

1. Replacement of the receptor-binding domain with a single-chain antibody

The first approach involved the replacement of the wild type receptor binding domain (RBD) of the envelope with a targeting moiety. The function of the RBD is to specifically bind the cellular receptor, and we have previously replaced the RBD with sequences encoding heregulin, a natural ligand for HER2/HER3 and HER2/HER4 heterodimeric receptors (Han *et al.*, 1995). The crystal structure of the MLV envelope has recently been determined (Fass *et al.*, 1997), and consists of a polypeptide chain partitioned structurally into a conserved immunoglobulin-like antiparallel beta-sheet framework. Noting the structural similarity between the RBD and immunoglobulins, we have pursued the strategy of replacing the RBD with a single-chain antibody directed against HER-2. HER-2, which belongs to the family of epidermal growth factor receptors, is over-expressed in about 30% of all human breast cancer cells; hence its use may be helpful in assessing the feasibility of using targeted RCR vectors for gene therapy of breast cancer.

Plasmids encoding single-chain antibodies (scFv) against HER-2 were generously provided by Dr. Michael Press and Jinha Park (University of Southern California). One of these scFv sequences in particular was selected, based on its high affinity and ability to trigger receptor-mediated endocytosis upon binding. With the use of the overlap extension PCR technique (Horton *et al.*, 1989), we successfully constructed two RCR vectors displaying this anti-HER2 scFv, one of which consists of a simple replacement of the RBD within the surface (SU) subunit of the envelope (ZDAH-emd) and the other which consists of total replacement of the SU subunit with direct fusion of the scFv to the transmembrane (TM) subunit (ZBAH-emd), and tested whether these modifications would ablate binding of the vector to its normal receptor while re-directing its binding to HER2-expressing breast cancer cells.

2. Insertion of the IgG-binding domain of protein A

The second approach, originally mentioned in the revised proposal, involves insertion of sequences encoding the immunoglobulin G (IgG)-binding domain ("Z domain," Nilsson *et al.*, 1987) of *Staphylococcus aureus* Protein A into the envelope gene. The site of insertion in this RCR vector construct, ZD-A-GFP, was the proline-rich region (PRR) or "hinge" of the envelope surface (SU) subunit. Insertion of exogenous sequences into the hinge region has been shown to not significantly hinder the native function of the envelope in mediating cell entry via the normal virus receptor (Wu *et al.*, 1998). The Z domain is the antibody-binding region of Protein A, and has a strong affinity for the Fc region of various mammalian IgGs, hence its presence of the Z domain on the vector surface would allow the binding of tumor-specific antibodies to the vector and thereby mediate specific binding of the vector to tumor cells via the antibody. It has previously been reported that targeted entry of replication-defective retroviral vectors can be achieved through the use of such chimeric retroviral envelope proteins containing the Z domain sequence of Protein A (Ohno *et al.*, 1997), and this strategy could enable the modular use of antibodies of various specificities for targeting entry of ZAPd-GFP into particular cell types.

Ecotropic vs. amphotropic envelopes

In initial studies, an additional safeguard employed to further minimize the risk of uncontrolled virus spread was the use of an RCR vector derived from ecotropic (i.e., murine species-specific) Moloney murine leukemia virus (MLV), ZAPd-GFP, which also carries a green fluorescent protein (GFP) reporter gene linked to the virus genomic sequences via an internal ribosome entry site (IRES). The envelope of ecotropic MLV cannot bind to human cells and hence cannot

mediate infection of human cells in its wild type form. Therefore, only if successful targeting occurred via the targeting moiety would infection of human cells be possible, and in this case such infection should be specific for breast cancer cells. Even if recombination occurred which somehow deleted the targeting ligand and reconstituted the wild type envelope, this would simply result in a virus that is non-infectious for human cells.

In later studies, we also employed RCR vectors in which the viral *env* (envelope) gene sequence was replaced with that of the amphotropic strain of MLV, which can bind to a common phosphate transporter (PiT-2) that is ubiquitously expressed on many cell types and highly conserved throughout evolution, and hence mediates broad host range tropism over many species including human and mouse.

Construction of RCR vectors containing suicide genes

Initially the targeted RCR vector constructs contained a GFP marker gene for ease of assay by fluorescence microscopy or flow cytometry. Subsequently, we also constructed RCR vectors containing suicide gene expression cassettes consisting of the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) linked to either the yeast cytosine deaminase (CD) or the E. coli purine nucleoside phosphorylase (PNP) suicide genes. These suicide genes were chosen due to their more potent bystander effect compared to Herpes simplex thymidine kinase (HSV-tk) and their smaller size, which appears to allow greater stability of the genomic sequence over serial replication cycles of the virus. These vectors have also been modified by replacement of the U3 promoter/enhancer region of the viral 5' long terminal repeat (LTR) with the cytomegalovirus (CMV) promoter enhancer; this modification allows higher levels of vector production upon initial transfection of the viral constructs into 293 cells. These suicide gene RCR vectors, designated ACE-CD and ACE-PNP, respectively, both contain the wild type amphotropic MLV envelope sequence, and were tested for their genetic stability over repeated serial infection cycles, and their ability to achieve efficient replicative gene transfer and suicide gene-mediated killing of cancer cells (see below).

Task 2. **Creation of RCR vector-producing cell lines (Months 8–14)**

Through completion of this task, we have obtained important results regarding the infectivity and efficiency of RCR vector-mediated transgene transmission.

Results:

Genomic stability of RCR vectors over serial infection cycles

We first examined the genomic stability of the untargeted RCR vector ZAPd-GFP, in which an internal ribosome entry site (IRES)-transgene cassette is positioned precisely between the retroviral *env* gene and the 3' untranslated region (UTR). While an insertion of 1.6 kb at the *env*/3' UTR position was lost immediately upon the first replication cycle, lengthened MLVs containing 1.2-1.3 kb *env*/3' UTR inserts proved to be highly stable, exhibiting replication kinetics that were only slightly attenuated compared to wild type virus, and maintained their genomic stability over multiple replication cycles, suggesting that these insertions had relatively small effects on replicative fitness (Logg et al, *J Virol* 2001; Logg et al, *Hum Gene Ther* 2001).

Eventually, multiple species of deletion mutants were detected simultaneously in later infection cycles; once detected, these variants rapidly dominated the population and thereafter appeared to be maintained at a relative equilibrium. Sequence analysis of these variants implicated preferred sites of recombination between short stretches of direct repeat homology as well as palindromic inverted repeats, and one instance of recombination with an endogenous provirus (Logg et al, *J Virol* 2001). Our results suggest that although lengthened genomes up to a certain size can be maintained over multiple replication cycles, preferential deletions that approximate the wild type genome length confer a strong selective advantage.

Breast cancer cell lines producing anti-HER2 scFv-targeted RCR vectors

Viral supernatants for infections were prepared by transient transfection of 293T with the respective scFv-containing RCR vector plasmids. Viral supernatant was harvested 48 hours after transfection, and filtered to remove cell debris prior to use in infection experiments. The target cells were the human breast cancer cell line MDA-MB-453 overexpressing HER-2 to test the tissue specificity of the viruses. To enhance the efficiency of infections we have used both lipofectamine and polybrene to facilitate retroviral entry. Lipofectamine is a cationic liposome which has been shown to mediate retrovirus infection of cells lacking the homologous viral receptor (Innes *et al.*, 1990; Porter *et al.*, 1998).

After initial infections, the MDA-MB-453 cells were passaged every 5 days for a period of 25 days and analyzed for GFP expression by FACS. Both vectors showed very low levels of infectivity suggesting that the scFv in place of the wild type RBD was not capable of mediating efficient entry and infection of target cells.

***In vitro* evolution of more efficient forms of targeted envelopes**

Due to the low infectivity of ZDAH-emd and ZBAH-emd, they were coexpressed with vesicular stomatitis virus G coat protein (VSV-G) and used for new infections to MDA-MB-453 cells. Previous studies have shown coat proteins from other viruses can be used to pseudotype MLV. In particular, VSV-G can encoat MLV cores efficiently and mediate transduction of a wide variety of cell types from many species (Emi *et al.*, 1991; Manning *et al.*, 1998).

The RCR vector plasmids were individually co-transfected into 293T cells along with a plasmid encoding VSV-G, and viral supernatant was harvested as previously. Thus, VSV-G would be co-expressed with scFv-envelope during the first-round infection, but as the VSV-G sequence is not part of the RCR genome itself, it will not affect subsequent spread of the scFv viruses.

Coexpression of VSV-G with these vectors allowed the highly efficient initial infection of MDA-MB-453 cells. These infected cells were then co-cultivated with uninfected cells for a long period, and thus the constantly released viruses from infected cells can bind and enter uninfected cells at a higher frequency. Since proliferation of the virus will depend on its ability to enter the cells efficiently, and retroviruses have a very high rate of mutation due to the relatively low fidelity of reverse transcriptase, we hypothesized that over time propagation of the pseudotyped virus would eventually give rise to mutations in the chimeric scFv-envelope that might allow more efficient breast cancer cell-specific virus entry by molecular evolution.

Genomic stability of Z domain insertion over multiple serial infections

The ability of the ecotropic, Z domain-targeted GFP-encoding vector ZD-A-GFP to transmit the transgene over multiple cycles of infection in culture was examined in the same manner as that described previously for the untargeted parental vector ZAPd-GFP. The cell line used was NIH3T3, and no antibody was employed. Our aim in this experiment was to determine the effect of the insertion in the proline-rich hinge region of the envelope on vector replication and transduction efficiency, independently of targeting. Low initial transduction levels were observed upon initial infection and over the first one or two serial passages, suggesting that the insertion significantly compromised the replicative ability of the virus. Subsequently, extremely high transduction levels comparable to the wild type parental virus were observed beginning in later infection cycles, suggesting that a reversion event might have occurred to delete the inserted sequences. Indeed, upon PCR analysis of virus sequences isolated from later passages, it was determined that the Z domain sequences had been deleted (Tai et al, *Hum Gene Ther* 2003). While genetic stability was improved by reduction of the overall size of the vector through use of a shorter IRES sequence derived from VEGF (vector ZV-A-GFP), deletion revertants still arose, suggesting that this type of insertion design will be useful for initial targeting of the virus to the tumor site, but that subsequent control of viral replication would require a different strategy.

Some of the above results have been reported in peer-reviewed publications with appropriate acknowledgment of DOD funding (Logg CR et al, *J Virol* 2001; Logg et al, *Hum Gene Ther* 2001; Tai et al, *Hum Gene Ther* 2003), and thus represent key reportable outcomes of this study.

Task 3. **Testing the tissue specificity of the virus in culture (Months 14–21)**

This task has been completed for both the scFv-targeted RBD domain replacement vectors as well as the IgG-targeted Z domain insertion vectors. In general, the domain replacement vectors showed extremely low levels of transduction, perhaps due to inefficient coupling of the targeted binding event mediated by the SU subunit replacement and the subsequent virus-cell membrane fusion event mediated by the TM subunit of the envelope. In contrast, the Z domain insertion vectors were able to successfully capture anti-HER2 monoclonal antibodies and mediate HER2-specific target cell binding and entry; however, additional host cell parameters must be investigated for this approach to be useful in human breast cancer cells (see below).

Results:

Low transduction efficiency of scFV-targeted RBD domain replacement RCR vectors

As reported above, MDA-MB-453 breast cancer cells were transduced with the ZDAH-emd and ZBAH-emd RCR vectors by co-pseudotyping with the VSV-G envelope on the first round of infection only, and these infected cells were maintained for more than a year to determine whether molecular evolution of targeted vectors would occur. Unfortunately, to date, we have been unable to isolate mutants of the targeted vectors that exhibit improved infectivity from these infected cells.

Specific binding of Z domain RCR vector/HER2 antibody complexes to HER2(+) cells

To assess whether the RCR vectors displaying the immunoglobulin-capture Z domain can specifically bind to HER2 overexpressing cells when complexed with an anti-HER2 antibody, we conducted virus-cell binding assays in the presence or absence of the antibody. For these binding assays, NIH/189, a NIH 3T3-derived murine fibroblast cell line stably expressing HER2, and the HER2-overexpressing human mammary carcinoma cell line SK-BR-3 were used as target cells, and the parental NIH 3T3 cells were used as negative control cells. Antibody-complexed or uncomplexed virion preparations were incubated with each cell population at 4°C to prevent endocytosis, excess unbound virus was washed away, and the bound virus was detected by FACS analysis using an antibody directed against the C-terminal portion of MLV *env* SU, an epitope that is still retained within the modified envelope proteins.

Strong enhancement of cell surface binding was consistently observed with RCR vectors pseudotyped by both ecotropic and amphotropic envelopes displaying the Z domain in the presence of anti-HER2 antibody on the HER2-expressing NIH/189 and SK-BR-3 target cells, compared to virus binding in the absence of antibody. Thus the display of anti-HER2 mAb tethered to Z domains in the PRR of MLV envelopes enhanced binding of the viral particles to HER2 antigen on the target cell surface (Tai et al, *Hum Gene Ther* 2003).

Antibody-enhanced transduction of RCR vectors

We sought to determine whether the previously observed HER2-specific antibody-mediated enhancement in target cell binding by Z domain-displaying RCR vectors could mediate a concomitant enhancement in their ability to transduce the HER2-positive murine NIH/189 and human SK-BR-3 cell lines. Additional human mammary carcinoma cell lines BT-474 and MDA-MB-453, which both overexpress HER2, as well as MDA-MB-231 which expresses only basal levels of HER2, were also tested for comparison. As tropism-modified envelopes frequently exhibit impairment of post-binding fusion activation, we also performed parallel experiments in which wild-type *env* was co-expressed along with the Z domain envelopes.

Target cell transduction was assessed by FACS analysis for GFP expression 48 to 96 hours after inoculation of parental control and recombinant targeted viruses complexed with anti-HER2 antibodies, as compared to uncomplexed viruses. As expected, the parental control RCR vector without the Z domain showed good overall transduction titers, but showed no difference in transduction efficiency in the presence or absence of antibodies, presumably because without the

Z domain, the virus failed to form complexes with the antibody. In contrast, an approximately 10-fold enhancement of NIH/189 target cell transduction was observed when the Z domain-containing envelope was co-expressed with wild-type ecotropic or amphotropic envelope in the presence of pre-complexed anti-HER2 antibodies, although this was not observed when the RCR vector was pseudotyped with the Z domain-containing envelope alone, and the enhanced transduction could be competed by addition of excess free antibodies at the time of infection. This result suggests that the immunoglobulin capture strategy was indeed effective in achieving enhanced transduction of these target cells in an antibody-specific manner (Tai et al, *Hum Gene Ther* 2003).

Interestingly, in stark contrast to the NIH/189 target cells, neither SK-BR-3, BT-474, MDA-MB-453, nor MDA-MB-231 can be specifically targeted by the anti-HER2 antibody complexed Z domain-displaying RCR vectors in the presence or absence of wild type amphotropic envelope. Thus, it appears that the antigen-antibody combination employed in these studies can achieve enhanced selectivity of infection for an engineered murine cell line expressing the target antigen but fails to achieve similar results using a human cell line that naturally overexpresses the target.

Our results therefore suggest that subtle differences in cellular parameters such as the type of receptor targeted and its level of cell surface expression, its cell surface localization, post-binding internalization process and compartment, etc., may significantly influence the efficiency of targeted vectors and should be thoroughly investigated; our results thus underscore the dangers of drawing conclusions from results obtained using artificially engineered target cell lines, which have been commonly employed in targeting studies, and thus have important implications for the design of vector targeting strategies in general.

These results have been reported in a recent peer-reviewed publication with appropriate acknowledgment of DOD funding (Tai et al, *Hum Gene Ther* 2003), and thus represent a key reportable outcome of this study.

Task 4. Transduction of human breast cancer xenografts *in vivo* (Months 21–36)

Due to the lack of effective targeting in human breast cancer cells despite the application of two different vector design approaches, we have been unable to completely fulfill our final aim of testing targeted RCR vectors in tumor models *in vivo*. However, in the course of these studies we have found that even untargeted RCR vectors are highly restricted in their replicative spread to rapidly dividing tumor cells *in vivo*, due to the intrinsic inability of MLV to infect quiescent non-dividing cells. Thus, even without envelope modification, RCR vectors exhibit tumor-selective replication, a characteristic that we would not have been able to discover without conducting animal studies proposed for this project using the untargeted control vector, and thus we have been able to definitively demonstrate the highly promising potential of RCR vectors for suicide gene therapy of breast cancer.

Results:

Highly efficient tumor transduction by replicative spread of RCR vectors

We first used untargeted RCR vectors expressing the GFP marker gene to determine the kinetics of replicative spread in a murine model of breast cancer.

Tumors were produced in athymic mice by the subcutaneous injection of 1.5×10^6 NMU rat breast adenocarcinoma cells. After 20 days the tumors, which had grown to 100-200 mm^3, were injected with 1.0×10^4 plaque-forming units of ZAPd-GFP in an 80 µl volume. Subsets of the mice were sacrificed and their tumors were surgically removed at 12, 22, 37, and 48 days following the vector injections. Following dissociation of the tumors into single-cell suspensions by mincing and incubation in collagenase, the tumor cells were examined by flow cytometry and immunohistochemistry for GFP expression.

The first tumor harvest (day 12) revealed minimal transduction in three out of four of the tumors examined. One of the tumors examined, however, exhibited a moderate transduction level, with approximately 8% of its constituent cells expressing GFP. By day 22, the number of tumor cells infected with the GFP-encoding virus had greatly increased. All 4 of the tumors removed from the mice at this time showed significant levels of transduction, averaging approximately one-third of the cell population. Comparable increases in the percentage of cells expressing GFP occurred by the two subsequent tumor harvests, on the 37th and 48th days after injection of the vector. By the 48th day, the average transduction level was approximately 75%, some tumors showing transduction levels approaching 100% (Logg et al. *Hum Gene Ther* 2001).

Tumor tissue taken at the 22 and 49-day time points was also examined by immunohistochemistry with an anti-GFP antibody to confirm expression of GFP within the tumors. While tumors removed at the earlier time point revealed patchy staining for GFP, with clusters of transduced cells adjacent to clusters of untransduced cells, low magnification views of tumor tissue taken at the later time point demonstrate highly efficient transduction throughout the tumor mass, with intense staining in almost every tumor cell (Logg et al. *Hum Gene Ther* 2001).

Thus, we have demonstrated that the untargeted control RCR vector ZAPd-GFP efficiently replicates and spreads within solid tumors, resulting in progressive spread of transgene to very high levels over time.

Spread to extratumoral tissues is not detectable even with untargeted RCR vectors.

In order to detect any spread of RCR vector outside the tumors that might have occurred, a variety of extratumoral tissues including spleen, lung, kidney, liver, and heart were harvested at the time of autopsy. High molecular weight DNA was harvested from each of these tissues and was used, along with DNA extracted from tumors, for PCR with primers specific for the GFP transgene. Amplification of serial dilutions of RCR plasmid demonstrated that this assay could detect as few as 140 copies of GFP in a background of approximately 100,000 equivalents (600 ng) of untransduced genomic DNA, representing an overall transduction level of about 0.14%. PCR analysis of tumor DNA taken from mice sacrificed 49 days after vector injection revealed the presence of the full-length GFP transgene, but none of the non-tumor tissues nor the mock-treated tumor sample exhibited any positive signals upon amplification. This unexpected result was also obtained upon examination of tissues harvested from animals sacrificed at earlier time points. These results suggest that extratumoral spread of the vector was minimal over the time course of these experiments (Logg et al. *Hum Gene Ther* 2001).

Inhibition of tumor growth by RCR vector-mediated suicide gene therapy

We tested the therapeutic potential of RCR vectors that transmit suicide genes as they spread through tumors *in vivo* in a breast cancer xenograft model. Amphotropic RCR vector (10^4 total dose/100 μl) carrying the yeast cytosine deaminase (CD) suicide gene or PBS vehicle control was injected into 500 mm^3 xenograft tumors previously established by subcutaneous inoculation of 2×10^6 MDA-MB-435 human breast cancer cells in nude mice.

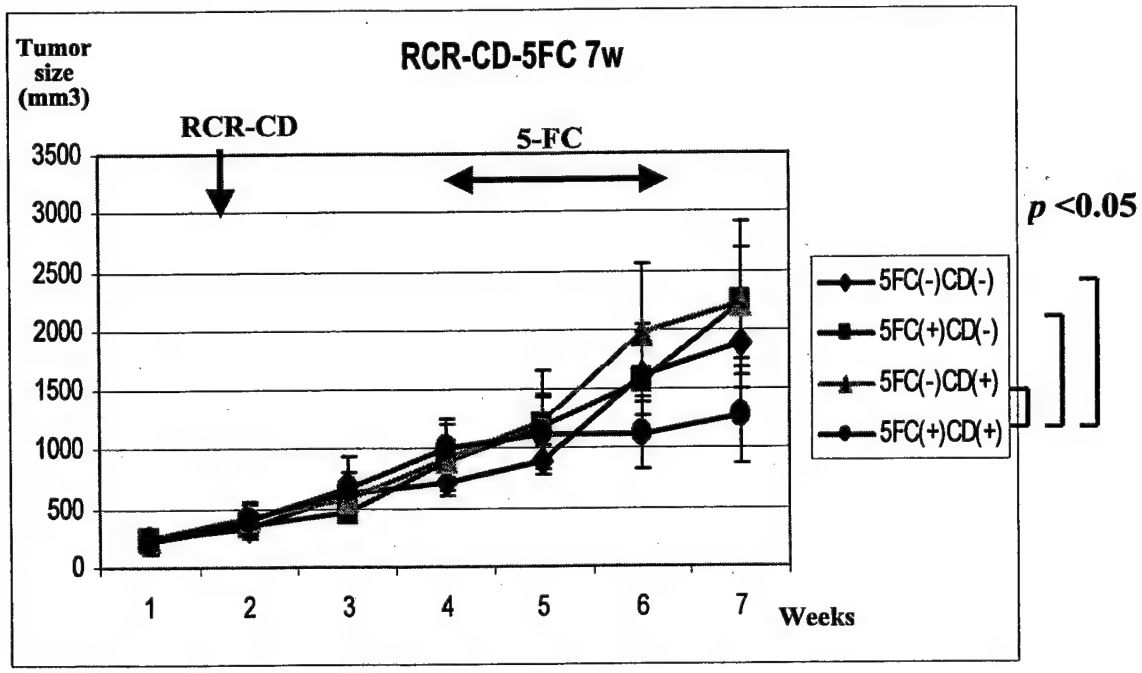
Two weeks after RCR vector injection, the animals subsequently received daily intraperitoneal injections of the pro-drug 5-fluorocytosine (5-FC; 500 mg/kg/day) or PBS vehicle control for 15 consecutive days. In RCR-infected cancer cells, the transgene-encoded yeast cytosine deaminase converts the non-toxic 5-FC pro-drug to the toxic metabolite 5-fluorouracil (5-FC), thereby killing the cell through intracellular chemotherapy.

Significant inhibition of tumor growth was observed throughout the period of pro-drug administration only in the RCR-CD vector + 5-FC treated group ($p<0.05$ compared to PBS only, RCR vector alone, and 5-FC pro-drug alone groups), suggesting that the efficient replicative spread of CD suicide gene-encoding RCR vectors through the tumor can achieve significant therapeutic benefit (see figure below).

The above results have been reported in a peer-reviewed publication (Logg et al, *Hum Gene Ther* 2001) and in abstracts and invited oral presentations at national meetings (see below) with appropriate acknowledgment of DOD funding, and thus represent key reportable outcomes of this study.

Growth inhibition of MDA-MB-435 tumors by suicide gene therapy with RCR vectors

RCR vectors expressing the cytosine deaminase suicide gene (RCR-CD) or PBS vehicle was injected into pre-established MDA-MB-453 breast cancer xenografts two weeks after tumor inoculation. Another two weeks after intratumoral vector or vehicle injection, each group of mice received daily intraperitoneal injections of the pro-drug 5-FC or PBS vehicle for 15 consecutive days. Tumor growth was monitored for each of the four treatment groups, i.e., PBS alone (5FC(-)CD(-)), 5-FC alone (5FC(+)CD(-)), RCR-CD alone (5FC(-)CD(+)), and RCR-CD plus 5-FC (5FC(+)CD(+)). The 5FC(+)CD(+) group vs. every other group shows significance at $p<0.05$.



III. KEY RESEARCH ACCOMPLISHMENTS

1. We have constructed several replication-competent retroviral (RCR) vectors targeted to breast tumor cells using two distinct types of modification to the envelope protein:
   - Replacement of the receptor-binding domain or the entire surface subunit of the envelope with an anti-HER2 single-chain antibody sequence targeted to breast cancer cells.
   - Insertion of the IgG-binding Z domain of protein A into the proline-rich hinge region of the envelope for modular conjugation of monoclonal antibodies targeted to breast cancer cells.
2. We have established producer cell lines for untargeted and targeted RCR vectors:
   - Using these producer cell lines, *in vitro* molecular evolution of more efficient forms of targeted envelopes was attempted but did not yield targeted vectors with enhanced efficiency.
   - Detailed studies of the genomic stability of untargeted and targeted RCR vectors were performed; to our knowledge, this is the first report of such studies and has resulted in two peer-reviewed publications.
3. We have performed cell culture studies to test targeted binding and transduction by RCR vectors encoated with envelopes containing the Z domain sequence:
   - Z domain-containing vectors show enhanced target-specific binding on both murine and human target cells.
   - With co-expression of wild type envelope, Z domain-containing RCR vectors show significantly enhanced antibody-mediated transduction on an artifical target cell line, but fail to show enhanced targeting on true human breast cancer cell lines.
4. I*n vivo* studies have demonstrated the intrinsic tumor selectivity of RCR vector replication even without envelope modification, and the efficacy of RCR-mediated suicide gene therapy in human breast cancer xenograft models.

IV. REPORTABLE OUTCOMES

Peer-reviewed publications:

(* please note that in publications 1 and 2 below, funding from this grant (#DAMD 17-99-1-9377) is acknowledged as Department of Defense Breast Cancer Research Program grant #BC980554, which was the original number of this grant application.

1. Logg, C. R., Logg, A. , Tai, C. K., Cannon, P. M., Kasahara, N.
   Genomic stability and natural selection of murine leukemia viruses containing insertions at the *env*-3' UTR boundary.
   Journal of Virology, 75: 6989-6998, 2001.

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   A uniquely stable replication-competent retrovirus vector achieves efficient gene delivery *in vitro* and in solid tumors *in vivo*.
   Human Gene Therapy, 12: 921-932, 2001.

3. Tai, C. K., Logg, C. R., Park, J. M., Anderson, W. F., Press, M. F., Kasahara, N.
   Antibody-mediated targeting of replication-competent retroviral vectors.
   Human Gene Therapy, 14: 789-802, 2003

Abstracts and Presentations

1. Logg, C. R., Logg, A., Tai, C.-K., Park, J., Press, M. F., Bochner, B. H., Anderson, W. F., Cannon, P. M., Kasahara, N.

   Targeting gene delivery to cancer cells using replication-competent retrovirus vectors.

   (Abstract selected for oral presentation at the 3rd Cold Spring Harbor Biotechnology Symposium on Vector Targeting Strategies for Therapeutic Gene Delivery, Cold Spring Harbor, New York, March 15-18, 2001.)

2. Logg, C. R., Logg, A., Tai, C. K., Wang, W., Anderson, W. F., Cannon, P. M., Bochner, B. H., Chen, T., Kasahara, N.

   Replication-competent retrovirus vectors targeted to cancer cells achieve highly efficient gene transfer to solid tumors *in vivo*.

   (Abstract selected for oral presentation at the 10th International Conference on Gene Therapy of Cancer, San Diego, California, December 13-15, 2001.)

3. Tai, C. K., Sazawa, A., Logg, C., Lee, R., Park, J., Press, M., Anderson, W. F., Cannon, P., Kasahara, N.

   Improving retroviral vectors for gene therapy of breast cancer.

   (Abstract for presentation at the Department of Defense Breast Cancer Research Program Era of Hope Meeting, Orlando, Florida, September 25-28, 2002.)

4. Kasahara, N.

   Replicative retroviral vectors: *teaching an old virus some new tricks*.

   (Invited oral presentation at the Breakthrough Advances in Vector Technology session, 10th annual meeting of the European Society of Gene Therapy, Antibes Juan-les Pins, France, October 12-16, 2002.)

Patents

Kasahara, N., Logg, C., and Anderson, W. F.

Gene Delivery System and Method of Use.

Provisional U.S. Patent Application No. 60/102,938
Conversion to full U.S. Patent Application No. 09/409,650

Cell lines, tissue or serum repositories developed

MDA-MB-453 human breast cancer-derived producer cells expressing anti-HER2 scFv-targeted RCR vectors ZDAH-emd and ZBAH-emd.

V. CONCLUSIONS

We have developed a stable, nondefective retroviral vector capable of high-level transduction both in culture and within solid tumors. This vector consists of a wild type Moloney MLV with an IRES-transgene cassette inserted between the 3' end of the *env* gene and the 3' UTR. We have shown that this vector possesses a high degree of stability during propagation and exhibits very high transduction efficiency when injected into solid tumors *in vivo*. As discussed above, many of these results have now been published in peer-reviewed journals (see Appendix).

There have been numerous published studies utilizing conventional non-replicating, or "replication-defective" retroviral vectors for *in vivo* gene delivery to tumors. In the large majority of these studies, the percentage of cells estimated to have been transduced was under 15%, despite the use of very high doses of vector or vector-packaging cells (Boviatsis *et al.*, 1994; Kruse *et al.*, 1997; Ram *et al.*, 1997; Short *et al.*, 1990; Smiley *et al.*., 1997). A recently reported Phase III clinical study of retroviral gene transfer into human brain tumors revealed that transduction of tumor cells occurred within only a few cell diameters of the tracts where vector-producing cells were injected, resulting in abysmally low transduction efficiencies, in some cases on the order of 0.01% (Rainov, 2000). In contrast, we estimate that the percentage of tumor cells transduced by our RCR vector *in vivo* approaches 100% (Logg *et al.*, 2001).

Since the general aim of cancer gene therapy is the destruction of malignant cells, the confinement of RCR vector spread to tumor cells would be of great utility. For this reason, we took steps toward engineering into the RCR vector the means to restrict vector replication to breast cancer cells, using two distinct strategies to re-target the binding tropism of the vectors via modification of the viral envelope structure.

One strategy for RCR vector targeting that we pursued has been to target breast cancer cells via replacement of the receptor binding domain (RBD) of the envelope by a single-chain antibody sequence (scFv) against HER2. Replacement of RBD by a scFv sequence destroys the ability of virus to bind its natural host receptor and moreover redirects its tropism. Our results suggest, however, that the introduction of a scFv into our RCR vectors greatly reduces overall transduction efficiency. This is in agreement with our own previous work, as well as that of others in the field of retrovirus targeting; in general, it has been found that efforts to re-target MLV envelope by incorporating scFv or ligand sequences results in loss of transduction efficiency (Kasahara *et al.*, 1994; Somia *et al.*, 1995; Marin *et al.*, 1996; Nilson *et al.*, 1996; Schnierle *et al.*, 1996; Valsesia-Wittmann *et al.*, 1996). It has been suggested that this may be due to an uncoupling of the binding and fusion steps, as infection does not occur even when binding to the new cell surface target is highly efficient.

To overcome this block, we pursued a molecular evolution approach to generate more efficient mutational variants of targeted envelopes. Since retroviruses have a very high rate of mutation, and impaired efficiency of viral entry by the HER2-targeted chimeric envelopes will exert a selective pressure that will favor variants capable of more efficient proliferation, we hypothesized that, over time, propagation of the targeted virus may give rise to second-site mutations in the chimeric envelope that allow more efficient breast cancer cell-specific virus entry. In order to facilitate this process, we first initiated a high level of infection in the human breast cancer target cells by co-expressing the VSV-G envelope, only during the first round of infection. Unfortunately, despite more than a year of continuous propagation in culture, no such variant quasi-species developed. To improve the odds of obtaining such variants in the future, it may be advisable to first generate a diversity of potential species by mutagenic PCR at the outset rather than relying on natural viral mutations to arise within the time frame of the experiment.

The second strategy entailed insertion of the immunoglobulin-binding Z-domain of protein A into the proline-rich region of the envelope to allow conjugation of the vectors to anti-HER2 antibodies. The use of antibody-antigen interactions as the basis for targeting may have a great benefit because re-targeting could be achieved simply by changing the complementary monoclonal antibodies.

Both amphotropic and ecotropic versions of the vector were constructed, and the site of insertion was the proline-rich "hinge" region in the surface subunit of the retroviral envelope. These modified envelopes could be efficiently expressed and incorporated into virions, and were capable of capturing monoclonal anti-HER-2 antibodies, which could in turn mediate efficient binding of the virus-antibody complex on human breast cancer cells and murine target cells stably overexpressing HER-2. However, although it has been reported that insertion of exogenous sequences into the hinge region does not significantly hinder the native function of the envelope in mediating cell entry via the normal virus receptor, we found that infectivity was markedly reduced in the Z domain/HER-2 antibody-targeted vectors, and co-expression of wild type envelope along with the targeted envelope was required to achieve efficient cellular entry. That this insertion was detrimental to viral replication was also underscored by its instability and rapid deletion over serial passage of the vector. In terms of its ability to achieve targeting, however, we noted that cell-specific transduction by both ecotropic and amphotropic Z-domain/HER-2 antibody-targeted vectors co-expressing wild type envelope was markedly enhanced on the murine target cells, and this enhancement could be competed by pre-incubation of the target cells with excess free antibodies. Interestingly, however, HER-2-expressing human breast cancer cell lines did not show antibody-mediated enhancement despite efficient cell surface binding of the targeted vectors, suggesting that target cell-specific parameters affect the efficiency of post-binding entry processes. In this regard, it should be noted that potentially critical parameters such as the type of receptor targeted and its level of cell surface expression, cell surface localization in relation to the natural virus receptor, post-binding internalization processes and intracellular fate of receptor-bound virions, etc., have yet to be rigorously investigated in the field of viral targeting.

As this approach would be limited *in vivo* by the availability of targeting antibodies upon serial replication after initial infection, we are also exploring the possibility of combining this approach with other mechanisms to limit subsequent viral infection events to the targeted cells. For example, after localizing the initial inoculum to tumors by antibody-mediated targeting, we can then employ tissue-specific transcriptional regulatory mechanisms to confine subsequent viral replication exclusively to breast cancer cells. We therefore intend to expand our studies to also achieve transcriptional targeting by incorporating regulatory elements from the breast cancer cell-specific lactalbumin or MUC-1 promoters into our RCR vectors. Expression studies have demonstrated that the proximal promoter region from either of these genes contains the regulatory sequences that specify transcription in human breast cancer cells (Abe and Kufe, 1998; Chen *et al.*, 1995). These regulatory sequences will be engineered into the long terminal repeat (LTR) promoter of the RCR vectors. In related studies, we have already demonstrated the feasibility of this approach by incorporating prostate-specific regulatory elements into the LTR, thus achieving prostate cell-specific transcriptional regulation (data not shown). We are also collaborating with Dr. Amy Lee (University of Southern California) to incorporate regulatory elements from the hypoxia-inducible GRP78 promoter into RCR vector LTRs as a more general mechanism for targeting viral replication to the hypoxic centers of solid tumors.

Finally, through the *in vivo* studies performed in this project, we have discovered that even untargeted RCR vectors exhibit properties which make them highly attractive as a tumor-selectively replicating gene transfer agent. MLV contains no nuclear localization signals and can only infect actively dividing cells, and as an integrating virus, the life cycle of MLV is non-cytolytic; this makes it unlikely that MLV will cause damage to post-mitotic normal cells as a direct consequence of viral replication. As reported above, we have found that intratumoral injection of as little as 10^4 total infectious units of RCR vector was found to be capable of spreading and transmitting an inserted transgene throughout entire solid tumor masses *in vivo*, achieving up to >99% transduction in breast cancer models, yet notably, systemic spread of vectors was undetectable by sensitive PCR assays in all normal tissues examined.

The use of RCR vectors for cancer gene therapy can thus allow highly efficient gene delivery from a small initial inoculum due to the amplification inherent in the replicative process. Although not intrinsically cytolytic, MLV-based RCR vectors can readily be engineered with suicide genes. Using this approach, we have achieved highly efficient killing of breast cancer cells in culture and in tumor models *in vivo*. Treatment of breast cancer xenografts with RCR vector-mediated suicide gene therapy resulted in significant inhibition of tumor growth throughout the period of pro-drug administration compared control groups. Interestingly, we now also have preliminary evidence that stable integration by MLV results in long-term persistence of viral infection, which follows cancer cells even as they metastasize to new sites, thus enabling multiple rounds of pro-drug administration to achieve further prolongation of therapeutic efficacy. Furthermore, RCR vectors thus confer permanent expression of viral proteins to the tumor cells with high efficiency; such stably and tumor-specifically expressed viral neoantigens can now be employed as a target for additional immunotherapeutic strategies. This makes the RCR vector system unique among the numerous oncolytic replicating virus systems now being tested as cancer therapeutic agents. The results we have obtained from these studies have thus suggested additional promising avenues which we are now pursuing.

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V. APPENDICES

Please see attached publications.

(listed in Reportable Outcomes section above, and Final Report Bibliography section below).

VI. FINAL REPORT BIBLIOGRAPHY/PERSONNEL LIST (see also Reportable Outcomes section above)

Peer-reviewed publications:

(* please note that in publications 1 and 2 below, funding from this grant (#DAMD 17-99-1-9377) is acknowledged as Department of Defense Breast Cancer Research Program grant #BC980554, which was the original number of this grant application.

1. Logg, C. R., Logg, A. , Tai, C. K., Cannon, P. M., Kasahara, N. 2001. Genomic stability and natural selection of murine leukemia viruses containing insertions at the *env*-3' UTR boundary. *J Virol*, 75: 6989-6998.

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Abstracts and Presentations

1. Logg, C. R., Logg, A., Tai, C.-K., Park, J., Press, M. F., Bochner, B. H., Anderson, W. F., Cannon, P. M., Kasahara, N. Targeting gene delivery to cancer cells using replication-competent retrovirus vectors.
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JOURNAL OF VIROLOGY, Aug. 2001, p. 6989–6998 Vol. 75, No. 15
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.15.6989–6998.2001

Genomic Stability of Murine Leukemia Viruses Containing Insertions at the Env-3′ Untranslated Region Boundary

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Received 17 January 2001/Accepted 4 May 2001

Retroviruses containing inserts of exogenous sequences frequently eliminate the inserted sequences upon spread in susceptible cells. We have constructed replication-competent murine leukemia virus (MLV) vectors containing internal ribosome entry site (IRES)-transgene cassettes at the *env*-3′ untranslated region boundary in order to examine the effects of insert sequence and size on the loss of inserts during viral replication. A virus containing an insertion of 1.6 kb replicated with greatly attenuated kinetics relative to wild-type virus and lost the inserted sequences in a single infection cycle. In contrast, MLVs containing inserts of 1.15 to 1.30 kb replicated with kinetics only slightly attenuated compared to wild-type MLV and exhibited much greater stability, maintaining their genomic integrity over multiple serial infection cycles. Eventually, multiple species of deletion mutants were detected simultaneously in later infection cycles; once detected, these variants rapidly dominated the population and thereafter appeared to be maintained at a relative equilibrium. Sequence analysis of these variants identified preferred sites of recombination in the parental viruses, including both short direct repeats and inverted repeats. One instance of insert deletion through recombination with an endogenous retrovirus was also observed. When specific sequences involved in these recombination events were eliminated, deletion variants still arose with the same kinetics upon virus passage and by apparently similar mechanisms, although at different locations in the vectors. Our results suggest that while lengthened, insert-containing genomes can be maintained over multiple replication cycles, preferential deletions resulting in loss of the inserted sequences confer a strong selective advantage.

Replicating retrovirus populations are characterized by a high degree of genetic change (6). This genetic diversity is the product of high frequencies of base misincorporations (4, 13, 40), rearrangements (7, 57), and both homologous (5, 18, 48) and nonhomologous (33, 58, 59) recombination events in the viral genome. Such genetic variability gives retroviruses the ability to adapt quickly to changes in selective pressures.

A variety of replication-competent retroviral vectors have been created by the insertion of heterologous sequences into full-length viral genomes. Such vectors have been constructed from several retrovirus species, including Rous sarcoma virus (RSV) (3, 29, 36), murine leukemia virus (MLV) (10, 28, 39, 49), spleen necrosis virus (12), human immunodeficiency virus (21, 27, 32, 55), and human foamy virus (42). In studies in which the structure of these lengthened viruses was examined subsequent to replication, the inserted sequences were usually found to have been partially or completely lost from the population within three or fewer passages through cultures of susceptible cells (12, 24, 28, 39, 42, 49). The tendency of retroviruses to rapidly delete insertions has been observed with various different insert sequences, indicating that the sequence requirements for efficient deletion are fairly permissive.

Previous studies that have analyzed in detail deletion mutants of nondefective retroviruses utilized RSV, which loses most its *src* coding sequence upon replication in culture (2, 31, 33, 58). However, differences in the stability of the RSV *src* gene in transformed versus nontransformed cells have been reported, suggesting that the presence of this oncogene may result in selective pressures on cultured viruses based not only on the virus's replicative fitness, but also on the cytoproliferative function of the protein encoded by this gene (2, 30). In this system, examination of virus stability in the absence of such selective pressures is therefore difficult.

We have recently shown that an internal ribosome entry site (IRES)-transgene expression cassette inserted at the *env*-3′ untranslated region (UTR) boundary in the MLV genome results in a fully replication-competent vector that can be used as a tool to efficiently and reliably transmit transgenes in single-passage mammalian cell culture (28a, 46). In the present studies, we used this system to examine the genetic stability of insert-containing MLVs and analyzed the effects of particular sequences in these viruses on the emergence of deletion variants during virus passage.

MATERIALS AND METHODS

Retroviral vector plasmid construction. An infectious Moloney MLV proviral clone was excised with *Nhe*I, which cuts once within each long terminal repeat (LTR), from plasmid pZAP (45) (kindly provided by John A. Young, University of Wisconsin) in order to eliminate flanking rat genomic sequences and recloned in the plasmid backbone of MLV vector g1ZIN to produce plasmid pZAP2. The region of the *env* gene from the unique *Nsi*I site to the termination codon was amplified by PCR and fused to the encephalomyocarditis virus IRES (22) amplified from plasmid pEMCF by overlap extension PCR (16), introducing the restriction sites *Bst*BI and *Not*I at the 3′ end. Plasmids g1ZIN and pEMCF were kindly provided by W. French Anderson, University of Southern California. The region from the *env* termination codon to the 3′ end of the 3′ LTR was also amplified by PCR, introducing *Not*I and *Afl*III sites at the 5′ and 3′ ends of the

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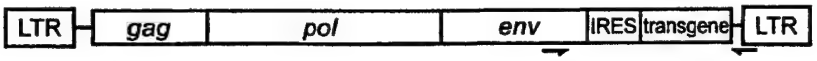
A.



B.

Vector	Transgene	Insert size	Sequence at 5' border of insert	Sequence at 3' border of insert
ZAPd-hygro	*hph*	1.55 kb	*env* IRES ...CGAGCCATAGATAACGTTACTGGC...	*hph* MLV 3' UTR ...CCGCGCCATAGATAAAATAAAAGATTTTA...
ZAPd-puro	*pac*	1.15 kb	"	"	*pac* MLV 3' UTR ...ACAAGTAGCGGCCGCGCCATAGATAAAA...
ZAPd-GFP	GFP	1.30 kb	"	"	GFP MLV 3' UTR ...ACAAGTAGCGGCCGCGCCATAGATAAAA...
ZAPm-GFP	GFP	1.30 kb	*env* IRES ...CGAACCGTGAACGCGTTACTGGC...	"	"
AZE-GFP	GFP	1.30 kb	4070A *env* IRES ...CGAGCCATGACGTACGTTACTGGC...	"	"

FIG. 1. Insert-containing viruses used in this study. (A) General structure of viruses, showing location of IRES-transgene cassettes within the MLV genome. Arrows indicate location of primers used in PCR amplification of proviral DNA of passaged virus. (B) Structural details of each virus, including sizes of IRES-transgene inserts and nucleotide sequences at 5′ and 3′ borders of insert. Nucleotides in bold show the position of the *env* stop codon. An 11-bp repeat sequence that flanks the inserts of ZAPd-hygro, ZAPd-puro, and ZAPd-GFP is underlined.

amplification product, respectively. A three-way ligation was used to insert this PCR product and the overlap extension PCR product into pZAP2 at its *NsiI* site and an *AflIII* site in the plasmid backbone, producing plasmid pZAPd. The puromycin acetyltransferase gene (*pac*) from plasmid pPUR (Clontech), the hygromycin phosphotransferase gene (*hph*) from plasmid pTK-hygro (Clontech), and the green fluorescent protein (GFP) cDNA (9) of plasmid pEGFP (Clontech) were each amplified by PCR and inserted into the *BstBI* and *NotI* sites of pZAPd, in frame with the authentic start codon of the IRES, producing pZAPd-puro, pZAPd-hygro, and pZAPd-GFP, respectively. All regions generated by PCR were verified by sequencing. A pZAPd-GFP-based construct in which an 11-bp repeat sequence flanking the IRES-GFP insert was eliminated and replaced by an *MluI* site was also generated by site-directed mutagenesis and designated pZAPm-GFP. An additional construct in which the Moloney MLV ecotropic envelope was replaced with the amphotropic envelope from 4070A was generated by overlap extension PCR and designated pAZE-GFP.

Cell culture and virus production. 293T (11), NIH 3T3 (20), and XC (51) cells were cultivated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Virus stock was produced by transfection of 293T cells using calcium phosphate precipitation with pZAP2, pZAPd-GFP, pZAPd-puro, or pZAPd-hygro (47). Virus-containing supernatant was collected 48 h following transfection and passed through 0.45-μm syringe filters before use.

Viral assays. Reverse transcriptase (RT) activity in supernatants of infected cell cultures was assayed as described previously (53). Quantitation of the reaction products was carried out using a Storm PhosphorImager (Molecular Dynamics). Virus titers were determined by the UV-XC syncytium assay (41).

Single-cycle infection with replicating viruses in culture. NIH 3T3 cells at 20% confluence in 6-cm dishes were infected with stock virus at a multiplicity of infection (MOI) of 0.0005. At 3, 5, and 8 days postinfection, the cells were examined by microscopy and split 1:5, and an aliquot of the cells was analyzed for GFP expression by flow cytometry as described below.

Multiple-cycle infections with replicating viruses in culture. NIH 3T3 cells at 20% confluence in 6-cm dishes were infected with stock virus at an MOI of 0.001. At 2 days postinfection, the cells were split 1:5, and in the case of ZAPd-GFP, aliquots were analyzed for GFP expression by flow cytometry as described below. At 4 days postinfection, 100-fold dilutions of cell culture supernatant were used to infect fresh NIH 3T3 cultures. At the 4-day time point, we subcultivated each culture for preparation of unintegrated proviral DNA and analyzed ZAPd-GFP-infected cells by flow cytometry. This cycle was repeated several additional times. Proviral DNA was extracted from each culture by a modified Hirt procedure (26).

Flow cytometry. NIH 3T3 cells were washed with phosphate-buffered saline, trypsinized, and collected by low-speed centrifugation. Cells were resuspended and analyzed with a Becton Dickinson FACScan fluorescence-activated cell sorter (FACS) using the FL1 emission channel to monitor green fluorescence.

Southern blot analysis of viral genomes. Hirt DNA was digested with *NheI*, separated by electrophoresis, and blotted onto nylon membranes. Probes were generated by [^{32}P]dCTP-labeled random priming of restriction fragments from each specific transgene or a common 2-kb *NheI-XhoI* MLV LTR-*gag* fragment. The blots were hybridized and washed under standard conditions and analyzed by PhosphorImager.

PCR analysis of viral deletion mutants. PCR amplification of Hirt DNA from infected NIH 3T3 cells was performed using upstream primers hybridizing to the ecotropic (in the case of ZAPd-hygro, ZAPd-GFP, and ZAPm-GFP) or the amphotropic (in the case of AZE-GFP) *env* gene and a common downstream primer hybridizing at the 3′ UTR-3′ LTR border. Upon electrophoresis, PCR products that were smaller than the expected size for full-length virus genomes were gel purified and sequenced.

RESULTS

Generation of lengthened MLV-based retroviruses. The encephalomyocarditis virus IRES linked to sequences encoding puromycin acetyltransferase, GFP, or hygromycin phosphotransferase was inserted into the ecotropic MLV genome, positioning the insertion immediately after the *env* termination codon. These plasmids were designated pZAPd-puro, pZAPd-GFP, and pZAPd-hygro and contained IRES transgene insertions of 1.15, 1.3, and 1.55 kb, respectively, positioned at the *env*-UTR boundary (Fig. 1). Infection of NIH 3T3 cells with ZAPd-puro and ZAPd-hygro stock virus conferred resistance to puromycin and hygromycin, respectively, and cells infected with pZAPd-GFP exhibited bright green fluorescence when observed by UV light microscopy, demonstrating that the vectors mediated functional expression of each transgene (data not shown).

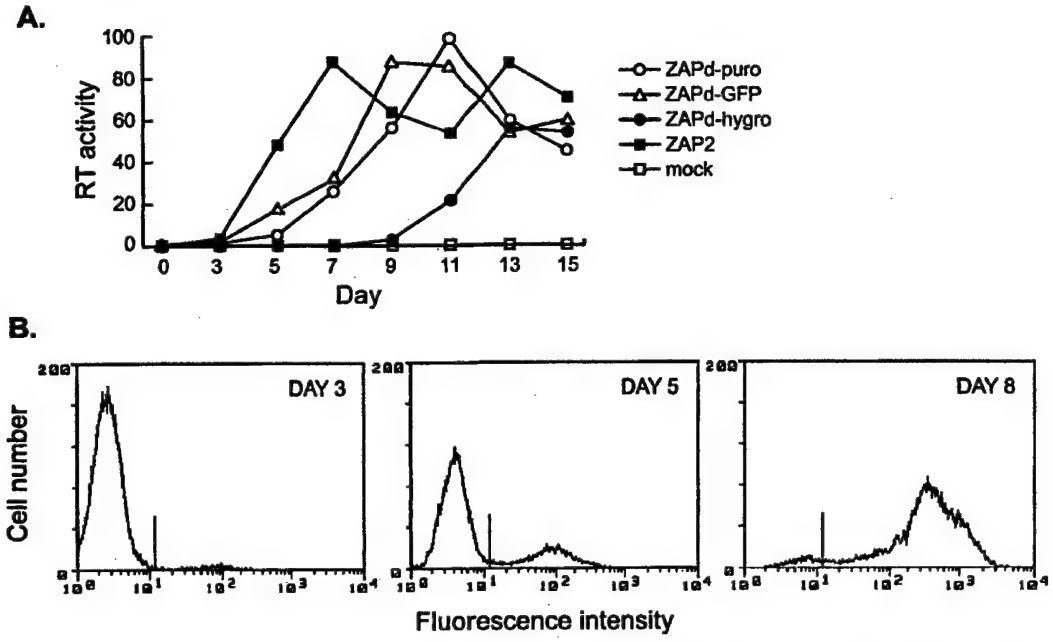


FIG. 2. In vitro replication kinetics of viruses. (A) NIH 3T3 cells were transfected with pZAPd-puro, pZAPd-GFP, pZAPd-hygro, or pZAP2 and passaged for 15 days. Culture medium was harvested from confluent cells every 2 days starting on day 3 and assayed for RT activity as described in Materials and Methods. RT activities are expressed in arbitrary units. Values are the means obtained from two independent experiments. (B) Spread of ZAPd-GFP through a single culture as detected by flow cytometry. NIH 3T3 cells were infected at an MOI of 0.0005 and examined at 3, 5, and 8 days postinoculation. Histograms show fluorescence intensity versus cell number for each day indicated.

Replication kinetics of insert-containing viruses through a single infection cycle. To examine the ability of ZAPd-puro, ZAPd-GFP, and ZAPd-hygro to replicate in cultured cells, we monitored RT activity in transfected cultures over a 15-day period. The parental wild-type virus, ZAP2, was used in parallel as a control. Both ZAPd-puro and ZAPd-GFP showed approximately the same lag period (3 days) as wild-type MLV prior to the appearance of detectable levels of RT activity, and thereafter exhibited a time course slightly attenuated compared to that of wild-type MLV (Fig. 2), suggesting that the insert-containing viruses replicated with moderately slower kinetics than wild-type virus. In contrast, ZAPd-hygro was greatly attenuated compared to wild-type MLV or the other insert-containing viruses, exhibiting a lag period of 9 days prior to the appearance of detectable RT. Thereafter, the rise in RT activity in the ZAPd-hygro-infected cultures was robust, suggesting that it may have derived from the exponential growth of an initially small revertant population. When propagated on NIH 3T3 cells, the titer of ZAPd-GFP reached 1.2×10^5 to 3.8×10^5 PFU/ml, while that of wild-type MLV on NIH 3T3 cells was 2.1×10^6 to 5.0×10^6 PFU/ml, indicating that the presence of the 1.3-kb IRES-GFP insert reduced production of infectious particles approximately 10-fold.

We also assessed the replication kinetics of ZAPd-GFP by following the spread of GFP through cells inoculated at low MOI. GFP fluorescence was detected in only a small percentage (~3%) of the cells 3 days postinoculation, while at 5 days, approximately one-quarter of the population exhibited fluorescence. By day 8, approximately 95% of the cells fluoresced (Fig. 2B), demonstrating that the virus transmitted the GFP marker gene with high efficiency.

Genetic stability of ZAPd-puro, ZAPd-GFP, and ZAPd-hygro upon replication through serial infection cycles. We serially reinoculated fresh plates of NIH 3T3 cells with cell-free ZAPd-puro, ZAPd-GFP, or ZAPd-hygro virus supernatants, using 100-fold dilutions of conditioned medium from the previous cycle for each subsequent infection, to examine stability over multiple replication cycles. No antibiotic selection pressure was applied during these infections.

Hirt DNA from each serially infected cell population, digested with *Nhe*I, which cleaves once within each LTR and thus yields a full-length linearized genome, was analyzed by Southern blot using probes specific for the corresponding transgene sequence, (Fig. 3A, C, and E, respectively) or a common probe for the 5′ LTR-*gag* region of MLV (Fig. 3B, D, and F).

The ZAPd-puro virus, which contains a 1.15-kb insert, showed no sign of deletion for the first six infection cycles, with only the full-length genome containing the insert sequence being detectable during this interval (Fig. 3A and B). At the seventh infection cycle, a variant population was detected at very low levels. This variant population hybridized to the LTR-*gag* probe (Fig. 3B) but not the *pac*-specific probe (Fig. 3A), and the genome size of this deletion mutant population appeared to be roughly similar to that of wild-type MLV. Over the subsequent five infections, the levels of this deleted population grew, while that of full-length ZAPd-puro decreased,

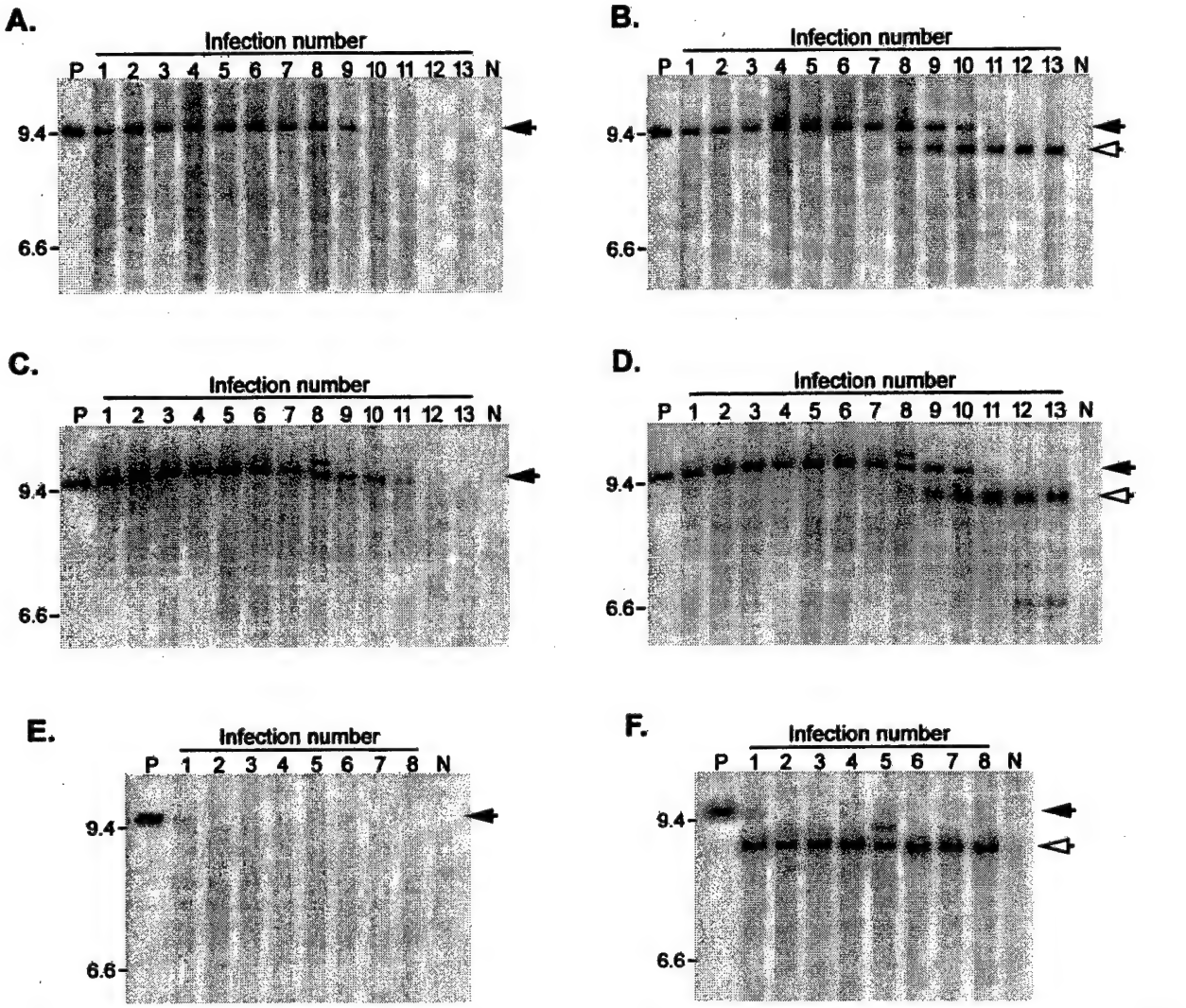


FIG. 3. Stability of insert-containing genomes over multiple serial infections. Viruses were subjected to repeated serial passage through NIH 3T3 cultures as described in Materials and Methods. Unintegrated proviral DNA was isolated from each virus passage, digested with *Nhe*I, and subjected to Southern blotting. (A and B) DNA from ZAPd-puro serial infections, using *pac*-specific probe (A) or MLV LTR-*gag* probe (B). (C and D) DNA from ZAPd-GFP serial infections, using GFP-specific probe (C) or LTR-*gag* probe (D). (E and F) DNA from ZAPd-hygro serial infections, using *hph*-specific probe (E) or LTR-*gag* probe (F). Lanes P, *Nhe*I-digested plasmid DNA encoding the corresponding virus. Lanes N, DNA isolated from mock-infected cells. Intact full-length provirus signals are indicated by solid arrows, and deletion mutants are indicated by open arrows. Expected full-length fragment sizes: ZAPd-puro, 9,437 bp; ZAPd-GFP, 9559 bp; ZAPd-hygro, 9851 bp. Positions of size standards are shown on the left (in kilobases).

indicating that loss of the insert imparted a replicative advantage.

Similarly, the full-length ZAPd-GFP signal was detected using either a GFP-specific probe (Fig. 3C) or the common LTR-*gag* probe (Fig. 3D) throughout more than eight serial infection cycles. This high level of stability was reproducibly and quite consistently observed through repeated experiments, each conducted with more than 10 serial passages. However, as observed with the ZAPd-puro virus, a progressive diminution in the full-length ZAPd-GFP signal was observed after the eighth infection cycle, corresponding with the progressive emergence of a variant virus population similar in size to wild-type MLV (Fig. 3D), and which was not detected by the GFP probe (Fig. 3C).

In contrast, proviral DNA from serial infections with ZAPd-hygro exhibited deletions from the first passage (Fig. 3E and F). Among the unintegrated proviral species produced upon the first infection, only a very small fraction proved to be full-length ZAPd-hygro (Fig. 3E), and by the second infection a deletion variant similar in size to wild-type MLV had completely outgrown the vector (Fig. 3F).

GFP fluorescence serves as a reliable surrogate marker for genomic stability of the ZAPd-GFP virus over multiple infection cycles. Based on the results of single-passage FACS anal-

ysis data and the extent of the deletions observed in ZAPd-GFP over serial passage, we hypothesized that the GFP reporter would greatly facilitate monitoring of virus replication and stability over multiple replication cycles. Therefore, we again performed serial infections with ZAPd-GFP, examining the cells by FACS at 2 and 4 days of each infection cycle. Only a small percentage of cells in each cycle expressed GFP by day 2 after inoculation (data not shown). However, the percentage of GFP-positive cells increased by day 4 of each cycle, indicating that GFP transduction was the result of progressive viral transmission rather than high initial levels of infection. The percentage of cells transduced by the virus at each day 4 time point, as determined by flow cytometry, is shown in Fig. 4A. Each serial infection up to the seventh or eighth cycle consistently generated transduction levels approaching 100% by day 4 postinoculation, after which the efficiency of GFP marker gene transmission was observed to decrease progressively, and almost no spread of GFP fluorescence was observed by the 15th cycle (Fig. 4A).

This progressive decrease in GFP transmission observed by FACS correlated closely with the emergence of the deletion mutant observed by Southern blot above, suggesting that the wild-type deletion mutant competes successfully with the insert-containing genome. This competition presumably occurred via superinfection resistance, as in the later infection cycles the maximum percentage of GFP-positive cells was not further increased beyond day 4 levels by additional cultivation of the cells (data not shown). The precise correlation between the loss of GFP expression and the loss of full-length forms also suggests that the level of GFP fluorescence can serve as a reliable surrogate marker for genomic stability and persistence of the ZAPd-GFP virus over multiple infection cycles.

Effect of repeat sequence deletion and envelope sequence replacement on genetic stability of lengthened MLV through multiple replication cycles. The IRES-transgene insert of ZAPd-GFP is flanked by an 11-bp repeat that might predispose the virus to a recombination event that would reconstitute the wild-type MLV sequence. We therefore constructed a variant of ZAPd-GFP, designated ZAPm-GFP, in which mutations were introduced into the upstream 11-bp repeat to eliminate homology with the downstream repeat sequence; these changes consisted of seven point mutations, including three silent mutations in the last three codons of *env* (Fig. 1). An additional variant of ZAPd-GFP in which the Moloney MLV ecotropic envelope was replaced with the 4070A amphotropic envelope was generated and designated AZE-GFP (Fig. 1). This vector, like ZAPm-GFP, lacks the upstream copy of the 11-bp repeat found in ZAPd-GFP.

ZAPm-GFP replicated with kinetics indistinguishable from those of ZAPd-GFP, as determined by transmission of the GFP marker through NIH 3T3 cultures after inoculation at low MOI (data not shown). Surprisingly, elimination of the upstream 11-bp repeat homology did not appear to prolong the stability of the virus over multiple infection cycles. Upon serial infection using the same protocol as above, ZAPm-GFP still showed progressively decreasing levels of day 4 postinoculation GFP fluorescence starting from infection cycle 8 (Fig. 4B), indicating that the same progressive loss of the full-length genome occurred from this cycle onward.

The AZE-GFP virus also showed efficient replication and

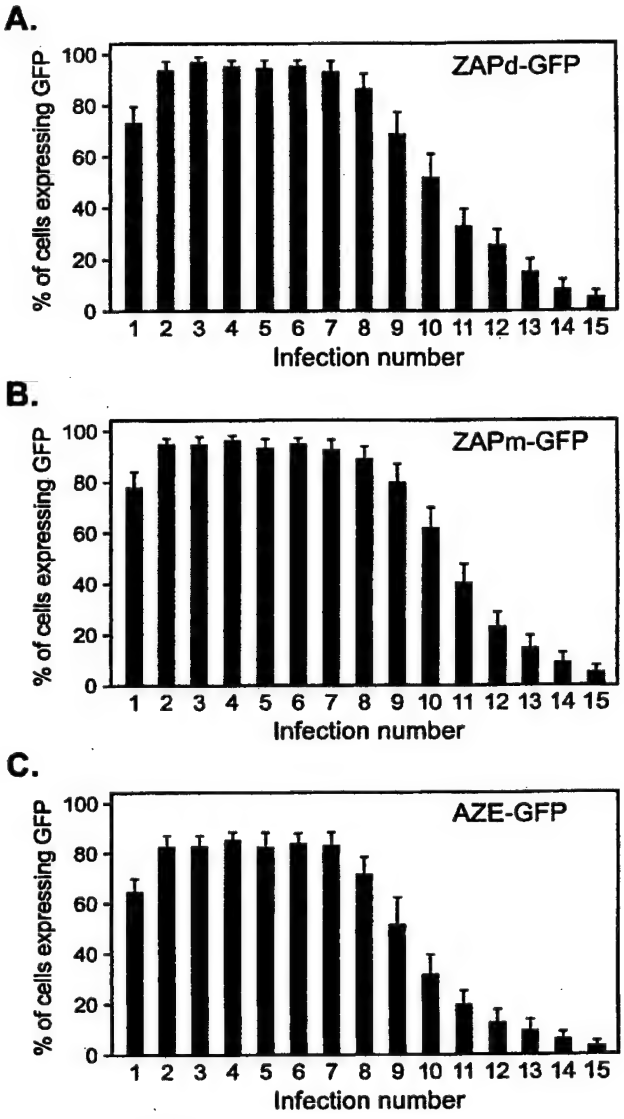


FIG. 4. Transmission of GFP by GFP-encoding viruses over multiple serial infection cycles. Each virus was serially passaged through multiple NIH 3T3 cultures as in Fig. 3. Four days after exposure to virus, each culture was examined for GFP expression by flow cytometry. Shown are the percentages of cells expressing GFP at each passage. Values were obtained from three independent experiments, and error bars represent standard deviations.

GFP transmission, demonstrating that the 1.3-kb IRES-GFP insertion at the *env*-UTR boundary is relatively stable even in the context of different upstream envelope sequences. However, the replication kinetics of AZE-GFP were found to be somewhat attenuated compared to ZAPd-GFP and ZAPm-GFP, and during serial infection experiments the percentage of GFP-positive cells after infection by the amphotropic virus only reached 80% by each day 4 time point up to cycle 7 (Fig. 4C), whereas its ecotropic counterparts had consistently reached around 95% by the same time point in previous experiments (Fig. 4A and B). In each of the first seven serial infection cycles, the percentage of GFP-positive cells did sub-

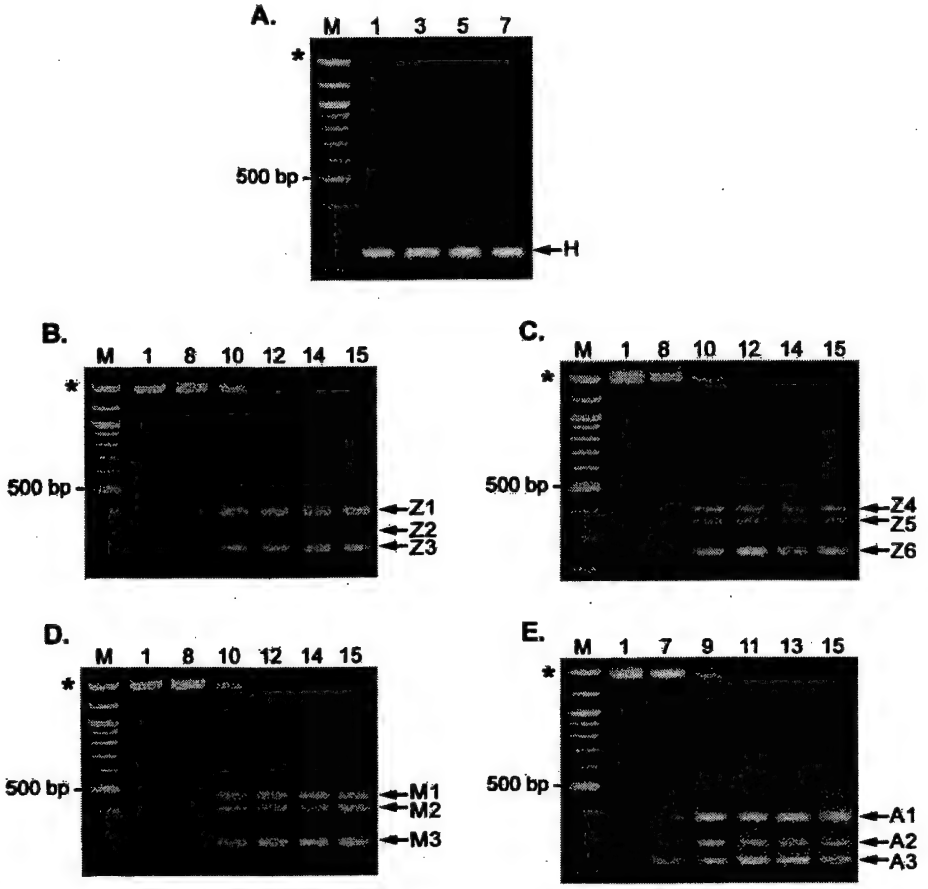


FIG. 5. PCR analysis of transgene insert region of serially passaged viruses. Hirt DNA from each indicated infection cycle was used as the template in PCR using an upstream primer specific for the appropriate *env* gene and a common downstream primer specific for the 3′ UTR-3′ LTR border region. (A) Amplification of passaged ZAPd-hygro. Overexposure of this gel (not shown) revealed the presence of a faint band of approximately the size expected for full-length ZAPd-hygro only in cycle 1. (B and C) Amplification of proviral DNA from two independent infection series using ZAPd-GFP. (D) Passaged ZAPm-GFP. (E) Passaged AZE-GFP. Lanes M, 100-bp molecular size markers. Asterisks indicate size of product expected for undeleted, full-length virus.

sequently reach 100% within the next 2 days, confirming that the lower percentage of fluorescent cells was due to delayed replication of full-length AZE-GFP and not to early emergence of deletion mutants (data not shown); however, the same time point (day 4) was used throughout for the serial infection experiments in order to preserve consistency in the assay. From cycle 8 onward, a progressive decrease in the percentage of fluorescent cells at each day 4 time point was observed, similar to that observed with the ecotropic viruses; this progressive decline in GFP fluorescence in later cycles could not be rescued by prolonged culture of each serially infected cell population, again indicating the overgrowth of deletion mutants.

Analysis of deletion mutants. We further characterized the deletion mutant populations that arose during the first infection cycle of ZAPd-hygro and after the seventh or eighth infection cycles of ZAPd-GFP, ZAPm-GFP, and AZE-GFP by PCR amplification and sequencing of Hirt DNA from each cycle. In the case of ZAPd-hygro, the PCR results indicate that the variant population observed on Southern blot consisted of a single major species of deletion mutant (Fig. 5A). In contrast, DNA from passaged ZAPd-GFP, ZAPm-GFP, and AZE-GFP revealed three major deletion species. However, none of these could be detected prior to the seventh or eighth infection cycles (Fig. 5B to E and data not shown), and all deleted forms appeared to emerge roughly simultaneously.

Sequencing of the amplified PCR products indicated that deletions occurred through recombination events between both direct and inverted repeats (Fig. 6A and B). Particular deletion patterns occurred repeatedly in independent experiments using different virus constructs (Fig. 6A, H/Z3/Z6 and Z1/Z4/M2/A1) and hence might represent preferred recombinational forms, while the other deletion species were unique to a single experiment or vector (Fig. 6A, A3, and Fig. 6B and C). One of the recurring deletion species, occurring in both ZAPd-GFP and ZAPd-hygro, exactly matched that of the wild-type *env*-UTR junction sequence (Fig. 6A, H/Z3/Z6). It is likely that this revertant derived from recombination between the 11-bp direct repeat sequences flanking the IRES-GFP insert (Fig. 1 and 6A), resulting in deletion of the entire 1,296-bp insert and reconstitution of the wild-type sequence. This species emerged rapidly and was the only one present after a

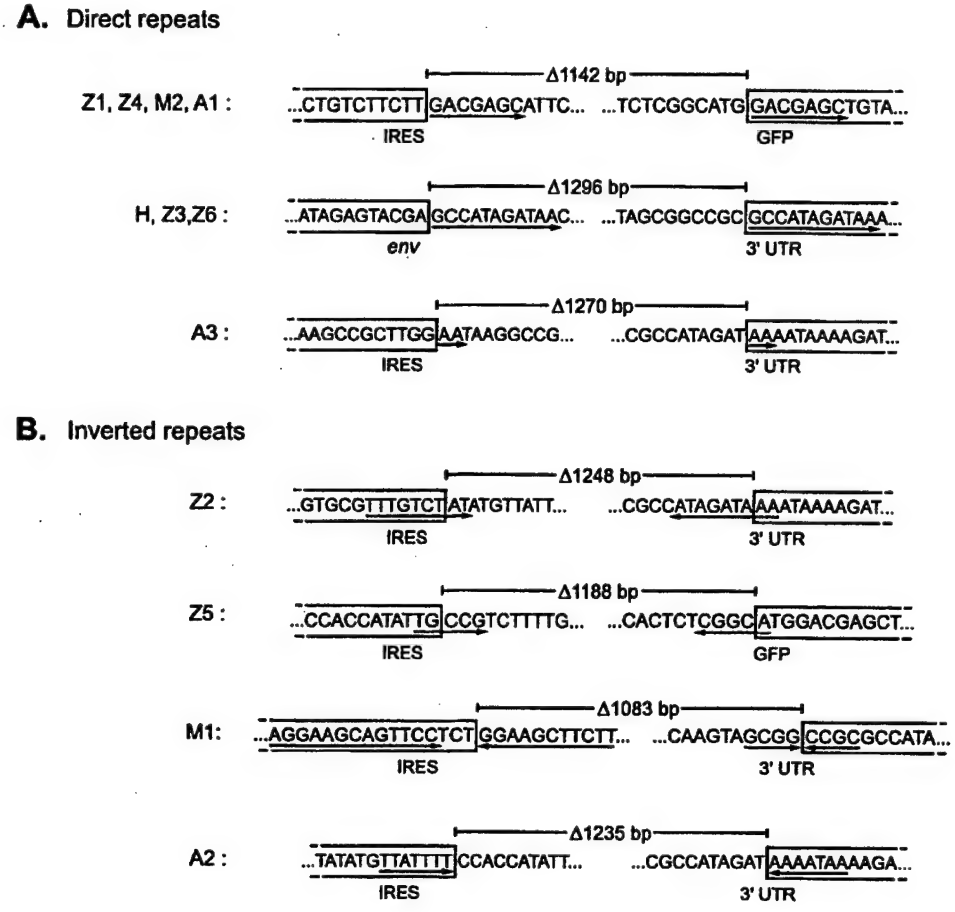


FIG. 6. Sequence analysis of deletion junctions of variants identified by PCR. Each of the PCR products shown in Fig. 5 was purified and sequenced. The sequences remaining in each deletion variant are boxed, and the intervening sequences deleted from the parental virus are unboxed. Lengths of deletions are indicated above each variant. (A) Deletion junctions of variants whose deletions were flanked by direct repeats in the parental genome. Direct repeats are underlined with arrows. (B) Deletion junctions characterized by inverted repeats in parental virus. Inverted repeats are underlined with arrows, and the orientation of each repeat is indicated by arrow direction. (C) Alignment of ZAPm-GFP variant M1 with Moloney MLV and endogenous virus MMX-CZ3 by the Clustal X program (23). Envelope stop codons are indicated in bold.

single passage of ZAPd-hygro but, in the case of ZAPd-GFP, emerged only after multiple replication cycles and was not the only species present. As the upstream copy of the 11-bp direct repeat had been eliminated in ZAPm-GFP and AZE-GFP, we did not expect to find precise reconstitution of the wild-type sequence, and indeed this did not occur.

Another deletion species emerged repeatedly in all three GFP-encoding viruses (Fig. 6A, Z1/Z4/M2/A1). In this variant, recombination occurred between 7-bp direct repeats, one of which was located in the IRES and the other within the GFP sequence, leading to the loss of 1,142 bp of the insert.

The remaining deletion species differed in sequence and

were not consistently observed to arise from experiment to experiment. Sequence analysis of one such variant species, A3, implicated recombination occurring between two dinucleotide repeats (Fig. 6A). Four other deletion species were associated with inverted repeats in the parental virus sequences (Fig. 6B). In three of these species, one copy of an inverted repeat was present on each side of the deletion junction: variant Z2 had a 1,248-bp deletion associated with 9-bp inverted repeat sequences, Z5 had a 1,188-bp deletion associated with 5-bp inverted repeat sequences, and A2 had a 1,235-bp deletion associated with 7-bp inverted repeat sequences, situated at the recombination breakpoints (Fig. 6B). The fourth inverted repeat deletion (M1) was associated with two complete palindromes with the potential to form hairpins aligned at each recombination breakpoint (Fig. 6B). The remaining variant species, M3, generated a PCR product approximating the wild-type MLV *env*-UTR sequence in size (Fig. 5D). However, sequence alignment analysis showed significant disparities between the M3 sequence and the parental Moloney MLV sequence, and a BLAST search of GenBank revealed extremely high homology with an endogenous mouse retrovirus sequence (56) (GenBank accession no. AF017530) (Fig. 6C), suggesting that it was derived by recombination of ZAPm-GFP with endogenous retrovirus present in the NIH 3T3 cells.

DISCUSSION

We have constructed a series of replication-competent MLVs containing insertions at the *env*-3′ UTR boundary to examine virus stability over multiple serial infection cycles. Our results suggest that an insertion at this position can be well tolerated as long as the insert size does not exceed a certain threshold or contain recombinogenic sequences. This construct design thus allowed us to examine the population dynamics of lengthened MLV genomes over repeated passage. Use of the IRES-GFP insert allowed us to track virus spread by flow cytometry, with loss of fluorescence serving as a reliable surrogate marker for genetic instability and the concomitant emergence of deletion mutants. In collaborative studies, we have also successfully employed a similar IRES-GFP insertion in feline leukemia virus (FeLV) to follow FeLV-A–to–FeLV-B conversion, suggesting that this strategy may prove to be generally useful in studies of retrovirus replication (Z. Chang, J. Pan, C. Logg, N. Kasahara, and P. Roy-Burman, submitted for publication).

On comparing replication-competent MLV vectors containing insertions between 1.15 and 1.55 kb long, we found striking differences in stability which correlated with differences in replication kinetics observed in single-cycle infections. ZAPd-hygro, which contains an insert of 1.55 kb, displayed the most attenuated replication kinetics. When this virus was propagated, it was almost completely overgrown after a single passage by a fully deleted revertant possessing wild-type MLV sequence. In contrast, ZAPd-puro and ZAPd-GFP, with 1.15- and 1.3-kb inserts, respectively, exhibited replication kinetics much closer to those of wild-type virus and retained their inserts for much longer periods. Presumably, the replicative fitness of ZAPd-puro and ZAPd-GFP relative to ZAPd-hygro account, at least in large part, for their greater stability, as they were likely better able to compete with deletion variants that arose during replication.

Sequence analysis of the deleted variants indicated that some of the deletions may have occurred through inter- or intramolecular template switching (25, 35, 54) between short direct repeat sequences within and flanking the transgene cassette. Recombination between an 11-bp direct repeat sequence that flanks the insertions of ZAPd-hygro and ZAPd-GFP resulted in exact reconstitution of wild-type MLV sequence. Unexpectedly, when variants of ZAPd-GFP lacking the upstream copy of the 11-bp repeat were serially passaged, no improvement in stability was observed.

One deletion species arising by recombination between 7-bp direct repeats within the insert occurred in all IRES-GFP-containing vectors. Notably, while sequence analysis revealed that the IRES-GFP cassette contains 77 pairs of perfect direct repeats of 7 bp or more, only this particular repeat pair was involved in formation of a deletion mutant. One possibility is that the sequence context of this specific 7-bp repeat might be particularly recombinogenic. Alternatively, as this repeat is spaced further apart than any of the other 77 pairs, the frequent occurrence of this deletion species may simply reflect a higher rate of recombination between homologous sequences that are spaced further apart (19), although this idea has recently been challenged (1). A third possibility is that, while other deletion species might have arisen through recombination at the other repeats, this particular species exhibits the largest deletion and its genome size is closest to that of wild-type MLV, allowing it to compete more effectively during virus passage. This particular deletion was also associated with recognition sequences for murine topoisomerases Ib and II in the parental virus, in which the cleavage sites were precisely aligned with the deletion endpoints. The significance of this association, however, is unknown.

We also observed frequent deletion between inverted repeats. This type of deletion has been proposed to occur when RT jumps over hairpins formed by such repeats in viral RNA during reverse transcription (33). Interestingly, of the many deletion junctions that have been characterized in previous studies of defective retrovirus vectors, the majority occurred between sites with directly repeated sequences, but remarkably few occurred between sites having inverted repeat homology (14, 25, 34, 35, 38, 52, 57, 59). In contrast, about half of the deletion species that we observed occurred by recombination at inverted repeats.

We observed one instance of deletion via homologous recombination with an endogenous polytropic retroviral sequence, which resulted in loss of the entire IRES-GFP insert. Similar "patch repair" of an exogenous virus by an endogenous sequence has been observed previously (8, 43), although in the earlier cases the repair was of a lethal deletion rather than a nonlethal insertion.

It is notable that these multiple species of deletion mutants detected in the ZAPd-GFP, ZAPm-GFP, and AZE-GFP infections were in each case undetectable over the first six to seven infection cycles and subsequently appeared to emerge simultaneously. Neither Southern blot nor PCR analysis revealed any major deletion intermediates during the serial infections, suggesting that these short deletion mutants were the only major species to arise and that these were generated

without a stepwise series of progressive deletions. While it remains possible that longer deletion intermediates were present but not amplified efficiently in this assay, this is unlikely, as the full-length IRES-GFP sequence could be amplified efficiently and showed a progressive loss of amplification signal beginning at cycle 7 or 8, consistent with the Southern blot results.

The lack of intermediate forms could be a consequence of recombination occurring at an early stage. If reversion was an early event, it occurred at low frequency, and it is likely that very few such deletion mutants were present from the first infection cycle. In the case of ZAPd-hygro, it is clear that revertants were present from the initial infection. However, for the other vectors, the advantage in replicative fitness of the various deletion mutants may not have been strong enough for these to have become apparent until many cycles had passed. An alternative, though not mutually exclusive, explanation is that at least some of the revertant species arose simultaneously in later cycles due to the requirement for some prior initiating event which resulted in increased rates of subsequent recombination. Previous studies have suggested that retrovirus recombination occurs within a distinct subpopulation (17) and that one predisposing factor might be the emergence of variant forms of RT that exhibit an increased frequency of template switching (37, 50).

As no external selection pressure was applied on any of the insert-containing virus populations, all deletion mutants presumably gained predominance through natural selection processes favoring those species that replicated most efficiently. Once detected, the major deletion mutant species all appeared to persist and gain dominance together. Presumably these multiple species, which are all similar in size, were collectively represented by the deletion mutant signal on Southern blot, which also showed no apparent intermediate forms. While the PCR analysis employed is not absolutely quantitative, the relative amounts of the amplification products from these deletion species would be predicted to change over multiple infection cycles if any particular species gained dominance over the others; however, this was not observed, suggesting that all three major deletion species could replicate with similar efficiency, and thus remained at an apparent equilibrium.

The fact that each of the deletion variants observed, using various pathways of recombination, had lost at least 84% of the IRES-GFP sequence indicates a strong selective advantage for either the natural genome length or the loss of insert sequences not contributing to efficient replication. While it has become clear that there is no absolute limitation on genome length within a certain range (15, 44), cumulative inefficiencies at multiple stages in the retroviral life cycle may result in a significant overall replicative disadvantage for viruses harboring exogenous sequences, and thereby subject such genomes to stringent limitations by the process of natural selection.

ACKNOWLEDGMENTS

We thank Pradip Roy-Burman and W. French Anderson for critical review of the manuscript.

This work was supported by a pilot project grant through the USC/Norris Breast Cancer Research Program, Department of Defense Breast Cancer Research Program grant BC980554, and NIH grant R01 CA85908. C.R.L. is the recipient of a Breast Cancer Research Project Fellowship through the Norris Comprehensive Cancer Center, and C.-K.T. is the recipient of a predoctoral fellowship from the Susan G. Komen Breast Cancer Foundation.

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HUMAN GENE THERAPY 12:921–932 (May 20, 2001)
Mary Ann Liebert, Inc.

A Uniquely Stable Replication-Competent Retrovirus Vector Achieves Efficient Gene Delivery *in Vitro* and in Solid Tumors

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ABSTRACT

A major obstacle in cancer gene therapy is the limited efficiency of *in vivo* gene transfer by replication-defective retrovirus vectors in current use. One strategy for circumventing this difficulty would be to use vectors capable of replication within tumor tissues. We have developed a replication-competent retrovirus (RCR) vector derived from murine leukemia virus (MuLV). This vector utilizes a unique design strategy in which an internal ribosome entry site–transgene cassette is positioned between the *env* gene and the 3′ long terminal repeat (LTR). The ability of this vector to replicate and transmit a transgene was examined in culture and in a solid tumor model *in vivo*. The RCR vector exhibited replication kinetics similar to those of wild-type MuLV and mediated efficient delivery of the transgene throughout an entire population of cells in culture after an initial inoculation with 1 plaque-forming unit (PFU) of vector per 2000 cells. After injection of 6×10^3 PFU of vector into established subcutaneous tumors, highly efficient spread of the transgene was observed over a period of 7 weeks, in some cases resulting in spread of the transgene throughout the entire tumor. MuLV-based RCR vectors show significant advantages over standard replication-defective vectors in efficiency of gene delivery both in culture and *in vivo*. This represents the first example of the use of an RCR vector in an adult mammalian host, and their first application to transduction of solid tumors.

OVERVIEW SUMMARY

Low efficiency of gene delivery by currently used viral vectors remains a significant barrier to the success of gene therapy of cancer. To examine the possibility of achieving superior transduction efficiency utilizing retroviral vectors that are capable of replication, we inserted an IRES–transgene cassette between the *env* gene and 3′ LTR of an infectious full-length murine leukemia virus (MuLV) clone. We found that the resulting vector replicated with kinetics similar to those of wild-type MuLV, and was stable through multiple serial passages in cultured cells. Injection of this vector into established subcutaneous tumors in mice resulted in highly efficient transmission of the transgene and, in some cases, transduction of entire tumor masses. These results demonstrate the potential utility of replication-competent retroviral vectors for cancer gene therapy.

INTRODUCTION

ADVANCES IN TECHNIQUES for gene transfer and expression have made feasible the treatment of cancer at the genetic level by introduction of exogenous genes into tumor cells (Anderson, 1984). Clinical trials utilizing various gene therapy strategies are underway for a variety of malignancies. However, all of these strategies require efficient gene transfer and this step has been a major impediment (Robbins *et al.*, 1998; Smith, 1999; Vile *et al.*, 2000). Despite the use of viral vectors such as retroviruses, gene transfer efficiency *in vivo* has generally been inadequate for achieving significant therapeutic benefit.

Defective murine leukemia virus (MuLV)-based retroviral vectors, which have been the most commonly used gene delivery vehicles in clinical gene therapy protocols (Robbins *et al.*, 1998), are incapable of secondary infection of adjacent cells because of the deletion of essential viral genes. More efficient

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transduction could be achieved if replication-competent retroviruses were used, as the virus would multiply after the initial infection event and each infected target cell would itself become a virus-producing cell. However, uncontrolled virus spread could result in adverse consequences, and the possibility of generating replication-competent retrovirus (RCR) during vector production has been a primary concern of gene therapy investigators (Cornetta *et al.*, 1991a).

Nevertheless, replicating forms of other virus species, including adenovirus (Bischoff *et al.*, 1996; Wildner *et al.*, 1999; Alemany *et al.*, 2000; Heise *et al.*, 2000), paramyxoviruses (Lorence *et al.*, 1988; Sinkovics and Horvath, 2000), herpesvirus (Walker *et al.*, 1999; Pawlik *et al.*, 2000), and reovirus (Coffey *et al.*, 1998; Norman and Lee, 2000) have been exploited for cytolytic treatment of cancer. Replicative retroviral vectors have also been proposed for use in gene therapy (Russell, 1994; Vile *et al.*, 1998), but there have been no previous studies reporting the development of RCR vectors for therapeutic applications. Although retroviruses are not cytolytic, the incorporation of a suicide transgene into an RCR vector could be used as a means to kill tumor cells, and would also serve as a safety mechanism to eliminate the vector after adequate levels of transduction are achieved. Furthermore, the initial rationale for use of defective retroviral vectors in cancer gene therapy would still hold true for RCR vectors, that is, MuLV-based vectors can transduce only cells that are actively dividing (Miller *et al.*, 1990), and since the majority of normal cells are quiescent, transduction would be relatively selective for tumor cells.

All previously described replicating murine retroviral vectors have contained transgene inserts, ranging from 100 to 1200 bp, in the U3 region of the 3′ long terminal repeat (LTR) (Goff *et al.*, 1981; Reik *et al.*, 1985; Stuhlmann *et al.*, 1989; Dillon *et al.*, 1991). However, vectors based on this design have proven to be quite unstable, as most of these earlier constructs began losing their transgene inserts from the first infection cycle (Goff *et al.*, 1981; Reik *et al.*, 1985; Stuhlmann *et al.*, 1989; Dillon *et al.*, 1991), and even nonreplicating retroviral vectors containing U3 inserts show a high frequency of recombination and deletion events (Mavilio *et al.*, 1984; Junker *et al.*, 1995). Thus, none of these previous replicating MuLV vectors has been sufficiently stable to be useful for efficient and reliable gene delivery *in vivo*.

We therefore sought to develop a stable, nondefective retroviral vector capable of high-level transduction both in culture and within solid tumors. Employing a unique construct design, we have developed an MuLV-based RCR vector that contains an internal ribosome entry site (IRES)–transgene expression cassette inserted precisely at the *env*-3′ untranslated region (UTR) boundary in the MuLV genome. We have previously utilized this type of vector to achieve delivery of the cell cycle regulator p27(kip-1) to osteoblasts *in vitro* (Smith *et al.*, 2000), demonstrating that this construct design produces a retroviral vector that can efficiently replicate and transduce mammalian cells in culture. Here we demonstrate that this vector is highly stable, being capable of replicating without observable deletions through multiple serial infection cycles in culture, and can achieve highly efficient gene delivery to solid tumors *in vivo*; hence this report represents the first use of such a nondefective retrovirus vector to achieve gene delivery in an adult mammalian host.

MATERIALS AND METHODS

Retroviral vector plasmid construction

An *Nhe*I fragment from plasmid pZAP (Shoemaker *et al.*, 1981) (generously provided by J.A. Young, University of Wisconsin, Madison, WI) containing the wild-type ecotropic Moloney murine leukemia virus (MuLV) provirus was recloned to eliminate flanking rat genomic sequences, producing plasmid pZAP2. Overlap-extension polymerase chain reaction (PCR) (Horton *et al.*, 1989) was used to fuse the 3′ end of the *env* gene to the encephalomyocarditis virus internal ribosome entry site (IRES) amplified from plasmid pEMCF, and the resulting plasmid was designated pZAPd. Insertion of the enhanced green fluorescent protein (GFP) cDNA (Cormack *et al.*, 1996) from plasmid pEGFP-N1 (Clontech, Palo Alto, CA) into pZAPd, in frame with the authentic start codon of the IRES, resulted in the final construct pZAPd-GFP. All PCRs were performed with *Pfu* polymerase (Stratagene, La Jolla, CA), and the integrity of the pZAPd-GFP construct was verified by sequencing. The prefix p is omitted when referring to virus derived from plasmid pZAPd-GFP.

Cell culture and virus production

Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, was used for culture for 293T (DuBridge *et al.*, 1987) and NIH 3T3 (Jainchill *et al.*, 1969) cell lines. NMU rat mammary carcinoma cells (Cohen, 1982) were grown in minimum essential medium supplemented with 10% fetal bovine serum. All cells were grown at 37°C under 5% CO_2. The calcium phosphate precipitation method was used to transfect 293T cells with either pZAP2 or pZAPd-GFP for transient production of virus. The conditioned medium was harvested 48 hr posttransfection and filtered through 0.45-μm pore size syringe filters prior to use in transduction assays. All *in vitro* infections were carried out in the presence of Polybrene (4 μg/ml; Sigma, St. Louis, MO).

Retroviral assays

To determine the kinetics of virus spread in culture, NIH 3T3 cells were transfected with either pZAPd-GFP or pZAP2, using LipofectAMINE 2000 (Life Technologies, Rockville, MD). Reverse transcriptase assays were performed on the culture supernatants as described previously (Telesnitsky *et al.*, 1995), followed by quantitation of reaction products with a Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Vector titers were determined by the XC syncytial assay (Rowe *et al.*, 1970) and are expressed as plaque-forming units (PFU) per milliliter.

Single cycle transduction with replicating vector

Virus vector stock at a multiplicity of infection (MOI) of 0.01 or 0.0005 was used to transduce NIH 3T3 cells at 20% confluence. On posttransduction days 3, 5, and 8, the target cells were examined by phase contrast and UV light microscopy using an Olympus IMT-2 inverted microscope (Olympus America, Melville, NY) with a 100-W mercury arc lamp and fluorescein filter cube. In some experiments, the transduced cells

were trypsinized at each time point and one-fifth was replated, while the remainder was analyzed by flow cytometry.

Multiple cycle transductions with replicating vector

Virus vector stock at a dilution of 1:1000 was used to transduce NIH 3T3 cells at 20% confluence. On posttransduction day 2, the cells were trypsinized, one-fifth was replated, and the remainder was analyzed for GFP expression by fluorescence-activated cell sorting (FACS) as described below. On posttransduction day 4, the cells were again analyzed by FACS, and the conditioned medium was harvested, diluted 1:100, and used for the next round of transduction on a fresh plate of NIH 3T3 cells. This procedure was repeated for several cycles.

Flow cytometric analysis

NIH 3T3 cells were trypsinized, pelleted, and resuspended in phosphate-buffered saline (PBS). Flow cytometric analysis was performed on a FACScan (Becton Dickinson, Franklin Lakes, NJ), using the FL1 channel to quantitate GFP-expressing cells.

Southern blot analysis

After *Nhe*I digestion and agarose gel electrophoresis, 10 μg of genomic DNA from NIH 3T3 cells or NMU cell tumors was transferred onto Hybond-N+ filters (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were hybridized with a random-prime [^{32}P]dCTP-labeled probe for GFP or the 5′ LTR-*gag* region of MuLV, washed at high stringency, and analyzed by PhosphorImager.

Analysis of vector spread in solid tumors in vivo

Tumors were established by the subcutaneous injection of 2×10^6 NMU rat breast adenocarcinoma cells into the anterior flanks of 8-week-old *nu/nu* BALB/c mice (Simonsen Laboratories, Gilroy, CA). Four weeks later, the tumors had grown to 1–1.5 cm^3, at which time they were injected with 6×10^3 PFU of vector. At regular intervals thereafter, subsets of the mice were humanely sacrificed, and their tumors were surgically removed. A portion of each tumor sample was immediately frozen in liquid nitrogen for later sectioning and immunohistochemical staining. The remaining portion of each tumor was used for FACS analysis, explantation, and isolation of genomic DNA for Southern hybridization. For immunohistochemistry, tumor sections were incubated overnight at 4°C with a 1:8000 dilution of GFP-specific monoclonal antibody (Clontech). Immunoreactivity was visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine as chromogen. All sections were counterstained with hematoxylin. For FACS analysis and explantation, cell suspensions from tumors were prepared by mincing of the tissue and incubation for 1–2 hr at 37°C on a rocking platform in Hanks' balanced salt solution containing collagenase type III (100 U/ml) and 3 m*M* $CaCl_2$. After removal of tissue fragments by passage through a cell strainer, dissociated cells were pelleted by low-speed centrifugation, resuspended in PBS, and either analyzed by FACS as described above or explanted into Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and grown at 37°C under 5% CO_2.

Detection of extratumoral spread of vector

Genomic DNA was extracted at autopsy from spleen, lung, kidney, liver, and heart tissue of ZAPd-GFP-injected animals. Six hundred nanograms of each DNA sample was used in a 50-μl PCR with PCR SuperMix (Life Technologies) and primers for the enhanced GFP. Five microliters of the reaction products was resolved on a 1% agarose gel and visualized by ethidium bromide staining. The detection sensitivity of this assay was determined by amplification of the GFP gene from serially diluted pZAPd-GFP plasmid in the presence of untransduced tissue genomic DNA. As an internal control for the amplification procedure, each DNA sample was used in PCR with primers that amplified a 500-bp target within the mouse β-casein gene. Tissues from the same organs were also dissociated and grown in explant cultures as described above.

RESULTS

Design and production of a unique MuLV-based RCR vector

As the U3–insert construct design utilized in all previously reported MuLV-derived replicative vectors apparently predisposes the vector to rapid loss of the inserted sequences, we hypothesized that insertion of the transgene into a less sensitive position might enhance the stability of the vector. To this end, we inserted a transgene expression cassette, consisting of the 0.6-kb internal ribosome entry site (IRES) from encephalomyocarditis virus (Jang *et al.*, 1988) and the 0.7-kb green fluorescent protein (GFP) marker gene, precisely between the *env* stop codon and a region of the 3′ untranslated region (UTR) that binds reverse transcriptase during viral DNA synthesis (Wohrl *et al.*, 1995); the resulting construct was designated pZAPd-GFP (Fig. 1A and B).

Vector stocks were prepared by calcium phosphate transfection of 293T cells with pZAPd-GFP. The transfected cells were examined by UV light microscopy 48 hr later and found to brightly express GFP fluorescence (data not shown), confirming that the IRES–GFP transgene cassette was functional. Conditioned medium containing ZAPd-GFP vector particles was harvested from the 293T cells 48–72 hr after transfection. The initial vector stock titer as determined by XC assay ranged between 3×10^3 and 4×10^3 PFU/ml.

The ZAPd-GFP vector replicates efficiently in culture

To determine whether the insert-containing ZAPd-GFP vector was capable of efficient replication in culture, we transiently transfected NIH 3T3 cells with pZAPd-GFP or pZAP2. Every 2 days thereafter for a 2-week period, the transfected cell culture medium was sampled and assayed for reverse transcriptase activity. On the basis of the resulting reverse transcriptase activity profiles, the replication kinetics of the ZAPd-GFP vector appeared to be somewhat attenuated compared with that of wild-type MuLV (Fig. 1C). The titer of ZAPd-GFP on a fully transduced culture of NIH 3T3 cells reached $1.5–3.6 \times 10^5$ PFU/ml. In contrast, the titer of the parental wild-type MuLV when propagated on NIH 3T3 cells under the same conditions reached $2.0–3.4 \times 10^6$ PFU/ml. This suggests that the presence

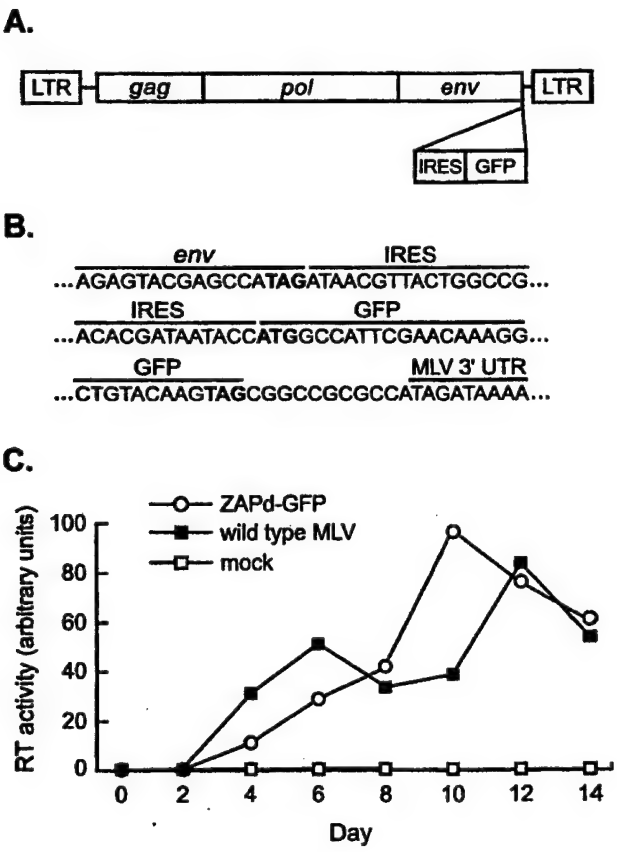

FIG. 1. (A) Structure of replication-competent vector ZAPd-GFP, showing site of insertion of IRES–GFP cassette into the wild-type murine leukemia virus genome. (B) Nucleotide sequence of ZAPd-GFP at the junctions between *env* gene and IRES, IRES and GFP, and GFP and MuLV 3′ untranslated region. Start and stop codons for the *env* and GFP genes within the junctions are in boldface. (C) *In vitro* replication kinetics of ZAPd-GFP and wild-type MuLV in NIH 3T3 cells over a 14-day period after transfection with pZAPd-GFP or pZAP2. Reverse transcriptase assays were performed with supernatants collected on the indicated days after transfection. Values represent means obtained from two experiments, and are expressed in arbitrary units. A control transfection was performed without adding vector plasmid (mock).

of the 1.3-kb IRES–transgene insert may have reduced virion production or impaired the replicative ability of the virus by approximately one order of magnitude.

The RCR vector mediates efficient in vitro *expression and transmission of the GFP transgene*

The reverse transcriptase activity profiles observed per se demonstrate that ZAPd-GFP can replicate but do not indicate whether the vector remained structurally intact and retained the IRES–GFP insert sequence through replication; nor does it indicate whether the transgene would be expressed from the virus. To answer these questions, we transduced NIH 3T3 cells with ZAPd-GFP at an MOI of 0.0005 and examined GFP expression in the culture by UV fluorescence microscopy at regular intervals thereafter (Fig. 2A). On day 3 postinoculation, GFP fluorescence was almost undetectable by microscopy; however, on day 5, GFP expression was detectable in about 10 to 20% of the cell population, and by day 9, almost the entire target cell population exhibited GFP fluorescence (Fig. 2B). Thus an initial inoculum of ZAPd-GFP on the order of 1 PFU per 2000 cells is sufficient to achieve highly efficient gene transfer to an entire cell population in culture.

These experiments were repeated using MOIs of 0.01 as well as 0.0005 and the percentage of GFP-expressing cells was quantitated by FACS analysis (Fig. 2C). At an MOI of 0.0005, the FACS results demonstrate a classic lag phase over the first 2–3 days, followed by a logarithmic increase in GFP expression as the ZAPd-GFP vector replicates through the culture, reaching the plateau phase as the entire cell population is transduced (Fig. 2C). The average burst size from a retrovirus-infected cell has been calculated to be on the order of 100 infectious virions (Dimitrov *et al.*, 1993), hence at an MOI of 0.01 (i.e., 1 PFU per 100 target cells) it is expected that a single replication cycle should be sufficient to achieve transduction of the entire culture. Indeed, at an MOI of 0.01, an almost immediate log-phase increase in GFP-positive cells is observed because of the higher initial input of vector particles, and the transduction kinetics are markedly accelerated as indicated by the transduction curve shifting to the left (Fig. 2C).

RCR vector-mediated GFP transmission is stable over multiple replication cycles

As the ability of the ZAPd-GFP vector to successfully transduce target cells during one or two rounds of replication had been established, we then sought to determine whether the RCR vector could stably retain and transmit the inserted transgene sequence through multiple replication cycles. Accordingly, we serially inoculated fresh populations of target cells and monitored spread of the GFP transgene through each culture. After initial inoculation of NIH 3T3 cells with ZAPd-GFP at an MOI of 0.001, the cells were examined by FACS on day 2, replated, and again examined by FACS on day 4 as described above. Prior to trypsinizing the cells for FACS analysis on day 4, the virus-containing culture medium was harvested, and a 100-fold dilution was used to inoculate a fresh population of NIH 3T3 cells. This procedure was repeated 6 additional times, using 100-fold dilutions of conditioned medium for each subsequent cycle, and GFP fluorescence was monitored by FACS on day 2 and day 4 of each cycle (Fig. 3A). Figure 3B shows the composite FACS results from three independent experiments. These results demonstrate that the percentage of GFP-positive cells increased between day 2 and day 4 during each individual replication cycle in a highly consistent and reproducible manner throughout, always reaching nearly 100% by day 4 of each cycle. This indicates that the ZAPd-GFP vector is highly stable in its ability to repeatedly transmit the GFP transgene throughout each fresh population of target cells.

The full-length RCR vector genome is retained during prolonged replication

To examine the structural stability of the ZAPd-GFP vector genome over the course of multiple replication cycles, and to rule out the possibility that the observed spread of the GFP

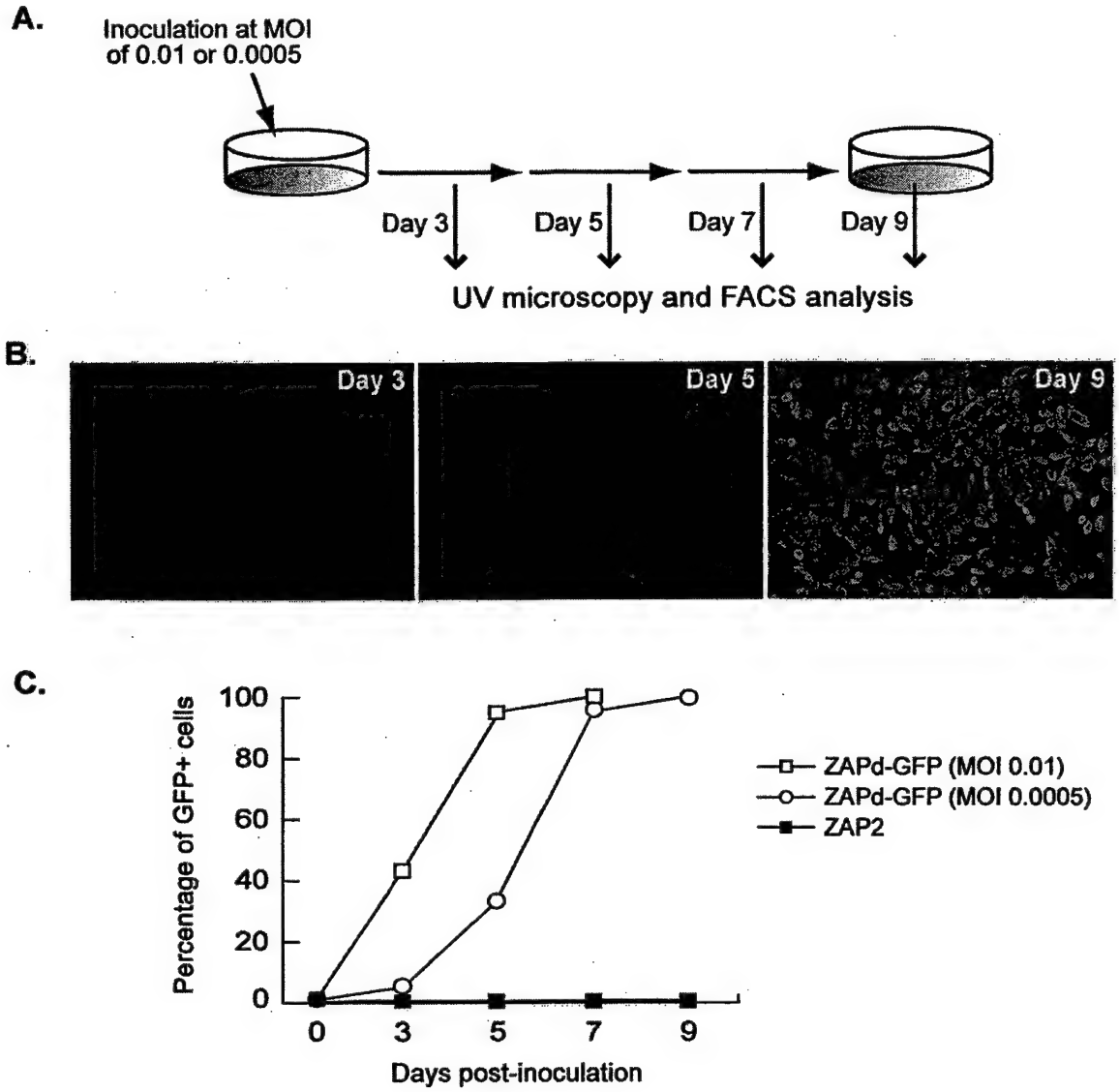

FIG. 2. Transmission of GFP transgene through a single culture infected with ZAPd-GFP. (**A**) Schematic diagram of experimental procedure. Cells were inoculated with ZAPd-GFP at an MOI of 0.01 or 0.0005, and 3, 5, 7, and 9 days later the cells were examined by fluorescence microscopy, passaged, and an aliquot was analyzed by flow cytometry for GFP expression. (**B**) Appearance of cells at 95–100% confluence by fluorescence microscopy immediately prior to passage after initial inoculation at an MOI of 0.0005 (original magnification, ×100). (**C**) Results of flow cytometric analysis. *y* axis, means of three independent experiments; *x* axis, days postinoculation.

transgene was primarily due to copackaging of a replication-defective vector retaining the GFP transgene and a replication-competent wild-type revertant lacking the transgene, we harvested genomic DNA from each serially infected NIH 3T3 cell population for Southern blot analysis. The genomic DNA samples were digested with *Nhe*I, which cuts once within each LTR and thereby releases the linear provirus, and probed with the GFP transgene sequence. Genomic DNA from all seven serial infection cycles demonstrated the presence of the full-length ZAPd-GFP provirus, predicted to be approximately 9.6 kb (equals approximately 8.3-kb wild-type MuLV genome plus 1.3-kb IRES–GFP transgene insert) (Fig. 3C). The presence of full-length ZAPd-GFP retaining the transgene, and the absence of deletion variants, was also observed by Southern blot analysis of unintegrated proviral DNA probed with the MuLV LTR-*gag* sequence (data not shown). Faint bands shorter than the full-length vector can be seen in some of the infection cycles, although it is unknown whether these represent deleted forms of the vector or nonspecific hybridization. The predominance of the full-length species in every infection cycle, however, suggests that serial GFP transmission was mediated primarily by the intact RCR vector.

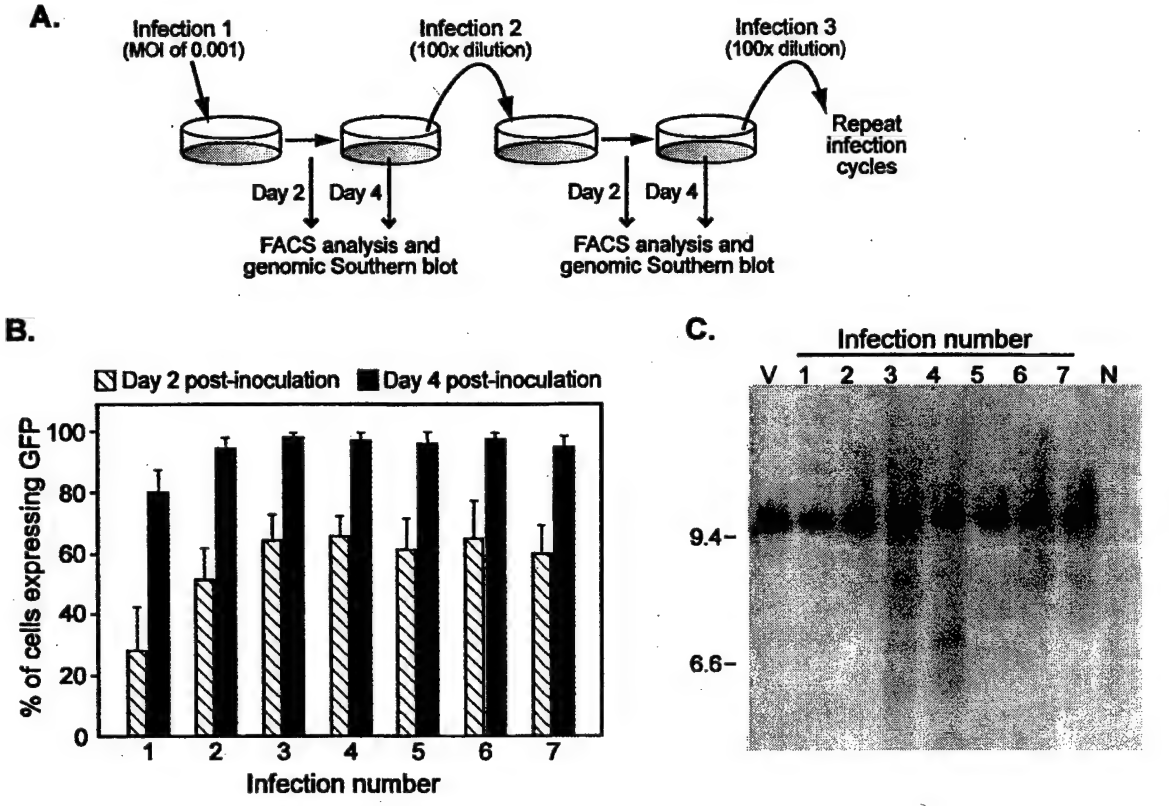

FIG. 3. Spread of ZAPd-GFP over multiple serial infections. (**A**) Diagram depicting the experimental procedure. NIH 3T3 cells were infected with ZAPd-GFP at an MOI of 0.001 (infection number 1). Six subsequent serial infections were conducted in which a 100-fold dilution of supernatant from each infection cycle was used to inoculate the subsequent cell population. Each population of infected cells was examined by flow cytometry and Southern blot hybridization of genomic DNA. (**B**) Summary of results of flow cytometric analysis. Shown are the percentages of cells expressing GFP in each infected culture 2 and 4 days after the inoculation step of each infection cycle. (**C**) Southern hybridization analysis of DNA from each of the seven infected cultures. Each DNA sample was digested with *Nhe*I prior to blotting and hybridization with the GFP cDNA probe. *Nhe*I cuts once in each LTR of the ZAPd-GFP provirus, generating a 9.56-kb fragment containing the transgene. Lanes: V, 30 pg of pZAPd-GFP plasmid DNA; 1–7, genomic DNA from each of the seven infected cultures; N, DNA isolated from mock-infected NIH 3T3 cells.

The RCR vector spreads through solid tumors and achieves highly efficient gene transfer in vivo

The ability of ZAPd-GFP to transmit its transgene through multiple serially infected cultures indicated that this vector might be able to spread within solid tumors *in vivo* and mediate transfer of the transgene into large numbers of tumor cells. To examine the ability of ZAPd-GFP to achieve efficient gene delivery in tumors, we injected the vector into preestablished mammary cancer xenografts in nude mice. Tumors were established by subcutaneous injection of 2×10^6 NMU rat mammary carcinoma cells; after 4 weeks, the tumors had reached volumes of 1.0–1.5 cm^3, and were injected with 6×10^3 PFU of ZAPd-GFP (Fig. 4A). Subsets of the mice were sacrificed and their tumors surgically removed 12, 22, 37, and 49 days after injection of the vector. After dissociation of the tumors into single-cell suspensions, the tumor cells were analyzed by flow cytometry for GFP expression (Fig. 4B). The first tumor harvest revealed minimal transduction in three out of four of the tumors examined. One of the four tumors examined, however, exhibited a moderate transduction level, with approximately 8% of its constituent cells expressing the GFP transgene. By day 22, the number of tumor cells infected with the virus had greatly increased. All four of the tumors removed from the mice at this time showed significant levels of infection, averaging approximately one-third of the cell population. Comparable increases in transduction levels occurred by the two subsequent tumor harvests, on the 37th and 49th days after injection of the vector. By the 49th day, the average transduction level was approximately 75%, with some tumors showing transduction levels approaching 100%. Explantation of dissociated tumor tissue and subsequent examination by fluorescence microscopy revealed that host-derived stromal cells within the tumor were also transduced by the vector (data not shown).

Tumor tissue taken at the 22- and 49-day time points was also examined by immunohistochemistry with an anti-GFP antibody to confirm expression of GFP within the tumors. While tumors removed at the earlier time point revealed patchy stain-

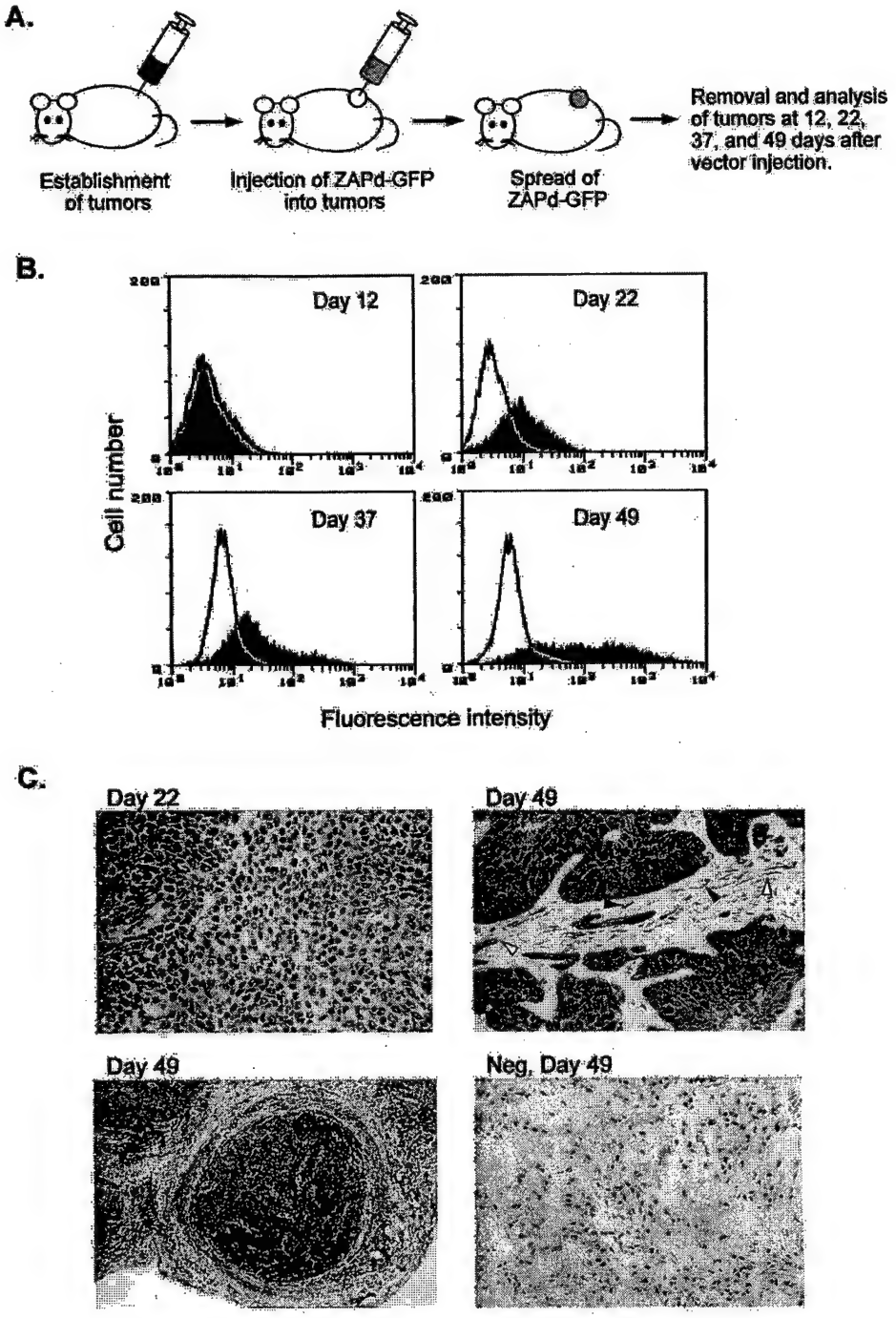

FIG. 4. Spread of ZAPd-GFP through solid tumors in mice. **(A)** Subcutaneous tumors, 1 to 1.5 cm^3 in volume, were injected with 6×10^3 PFU of ZAPd-GFP. Twelve, 22, 37, and 49 days after vector injection, tumors were removed from subsets of the mice and were analyzed for virus spread by flow cytometry and Southern blot hybridization. **(B)** Expression of GFP transgene in tumors. Tumor cells from each of the four time points were dissociated into single-cell suspensions and analyzed for GFP fluorescence by flow cytometry. Shaded histograms represent tumors injected with ZAPd-GFP, and open histograms represent untreated tumors. **(C)** Immunohistochemical staining of GFP in tumors injected with ZAPd-GFP. Tumors removed 22 and 49 days after vector injection were stained with a monoclonal antibody to GFP and counterstained with hematoxylin. *Top left*: Tumor removed 22 days after vector injection. *Top right* and *bottom left*: Tumors removed at 49 days. Open arrowheads indicate transduced fibroblasts and closed arrowheads indicate transduced endothelial cells. *Bottom right*: Negative control tumor removed 49 days after vector injection. Each panel represents a different mouse. Original magnification: Top left and bottom right, ×300; top right, ×400; bottom left, ×6.

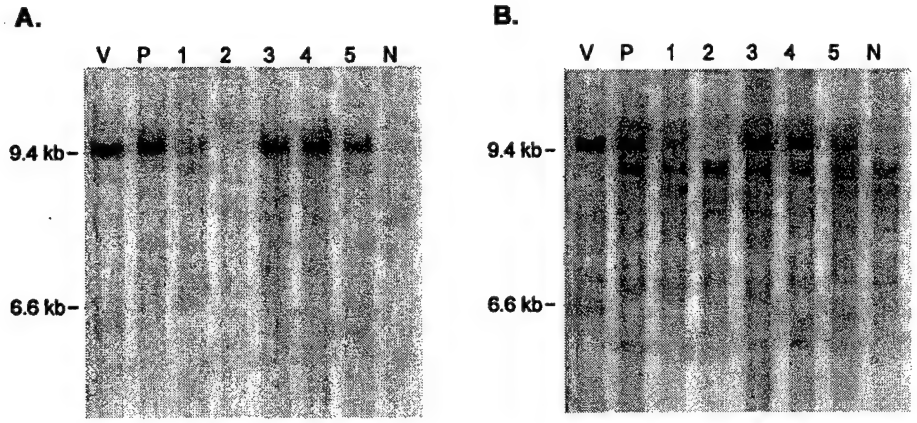

FIG. 5. Southern blot analysis of genomic DNA from tumors injected with ZAPd-GFP. Ten micrograms of DNA from each tumor was digested to completion with *Nhe*I. After blotting onto a nylon membrane, the fragments were hybridized to a random-primed radiolabeled probe for the GFP transgene or for the MuLV LTR-*gag* sequence. Lanes: V, 30 pg of pZAPd-GFP plasmid DNA digested with *Nhe*I; P, DNA from tumor originating from cells that were infected with ZAPd-GFP prior to their injection into mouse; 1 and 2, DNA from tumors removed 12 days after vector injection; 3, 4, and 5, DNA from tumors removed 37 days after vector injection; N, negative control tumor injected with virus-free supernatant. (A) GFP-probed blot; (B) MuLV LTR-*gag*-probed blot. Top band, full-length ZAPd-GFP; bottom band, nonspecific hybridization signal (also present in negative control tumor).

ing for GFP, with clusters of transduced cells adjacent to clusters of untransduced cells (Fig. 4C, top left), low-magnification views of tumor tissue taken at the later time point demonstrate highly efficient transduction throughout the tumor mass (Fig. 4C, bottom left). The latter samples show intense staining in almost every tumor cell, as well as distinct staining in fibroblasts and some endothelial cells (Fig. 4C, top right).

The full-length RCR vector genome is retained during prolonged replication in vivo

To confirm that integrated vector provirus was present in the tumor cells and that the GFP transgene had been transmitted as part of the intact vector, we performed Southern analysis on genomic DNA from tumors removed 12 and 37 days after vector injection. The only bands detected by the GFP probe were the size expected for full-length ZAPd-GFP (Fig. 5A), demonstrating the presence of integrated vector and that the high-level transduction shown by FACS analysis and immunohistochemistry, was the result of the transmission of the GFP transgene by intact ZAPd-GFP. The MuLV LTR-*gag* probe also hybridized to the full-length ZAPd-GFP band, as well as to a smaller band that is likely to represent an endogenous provirus present in the NMU cell line or the BALB/c genome, as the same band is present in genomic DNA from the untransduced negative control tumors (Fig. 5B). These results suggest that the transmission of the GFP transgene through the tumors during the course of the 37 days was mediated primarily or exclusively by the intact vector, and that deletion variants, if present, occurred only at levels undetectable in our analysis.

Extratumoral RCR vector spread is not at detectable levels under present conditions

To detect any spread of ZAPd-GFP outside the tumors that might have occurred, a variety of extratumoral tissues including spleen, lung, kidney, liver, and heart were harvested at the time of autopsy. High molecular weight DNA was harvested from each of these tissues and was used, along with DNA extracted from tumors, in PCR with primers specific for the GFP transgene. Amplification of serial dilutions of pZAPd-GFP plasmid demonstrated that this assay could detect as few as 140 copies of GFP in a background of approximately 100,000 equivalents (600 ng) of untransduced genomic DNA, representing a transduction level of about 0.14%. Figure 6 shows the results of PCR using samples taken from mice sacrificed 49 days after vector injections. DNA from the tumor injected with vector revealed the presence of the full-length GFP transgene, but none of the nontumor tissues or the mock-treated tumor sample exhibited amplification. This unexpected result was also obtained on examination of tissues harvested from animals killed at earlier time points (data not shown). Flow cytometric analysis of the same tissues 1 week after explanting also revealed the presence of the GFP transgene only in tumors (data not shown). These results suggest that spread of the vector originating from a total initial inoculum of 6×10^3 PFU was minimal over the time course of these experiments.

DISCUSSION

This study demonstrates that the insertion of exogenous sequences precisely between the *env* gene and 3′ UTR of MuLV results in a fully replication-competent vector that exhibits a high degree of genomic stability, compared with previously reported MuLV-based RCR vectors that contained inserts within the U3 region of the LTR. We chose this particular insert position because (1) the packaging signal extends past the start codon of the *gag* gene, and thus positioning a transgene just upstream of the *gag* gene would likely impair packaging efficiency; (2) the *gag* and *pol* coding sequences are initially translated as a single polypeptide that is then cleaved, thus posi-

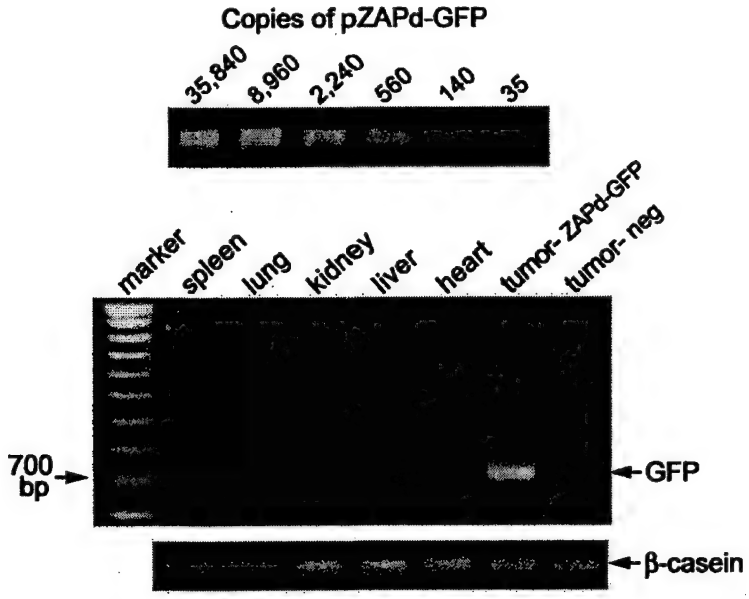

FIG. 6. Analysis of extratumoral spread of ZAPd-GFP by PCR amplification of GFP transgene from genomic DNA. The sensitivity of the assay was determined by amplification, using 4-fold serial dilutions of pZAPd-GFP as template in the presence of untransduced genomic DNA (*top*). Six hundred nanograms of DNA, extracted from tumors and various extratumoral tissues at the time of autopsy, was used in PCR analysis for the GFP transgene (*middle*). Shown are results with tissues taken from mice 49 days after injection of tumors with vector. Expected size of full-length amplification product is 730 base pairs. A 500-bp region of the mouse β-casein gene was amplified from the same samples as an internal control (*bottom*).

tioning a transgene between these coding sequences might interfere with proteolytic processing; (3) the 3′ end of *pol* overlaps with the 5′ end of *env*, precluding the insertion of a transgene between these genes; and (4) positioning the transgene, preceded by an IRES, just downstream of *env* would allow the transgene to be expressed from both the spliced *env* transcript as well as the unspliced genomic transcript. While the majority of previously reported U3–insert MuLV vectors, whose inserts were 100–1100 bp smaller than that of ZAPd-GFP, began losing their inserted sequences during the first replication cycle (Lobel *et al.*, 1985; Reik *et al.*, 1985; Stuhlmann *et al.*, 1989; Coulombe *et al.*, 1996), ZAPd-GFP was stable through continuous propagation over multiple replication cycles in culture, and furthermore, this vector efficiently replicated within solid tumors *in vivo*, leading to widespread delivery of the transgene at efficiencies significantly higher than those typically achieved using standard replication-defective retroviral vectors at much higher doses.

Most previous studies using standard replication-defective MuLV vectors for tumor transduction *in vivo* reported efficiencies of less than 10%, despite the use of large doses of vector or vector packaging cells, vector injection at multiple time points, or simultaneous injection of tumor and packaging cells (Short *et al.*, 1990; Yang *et al.*, 1996; Kruse *et al.*, 1997; Ram *et al.*, 1997; Smiley *et al.*, 1997). The low *in vivo* transduction efficiency of defective retroviral vectors has been attributed to the inability of the vector particles to diffuse from the site of injection. The results of clinical trials of retroviral gene transfer into brain tumors, in which intratumoral injection of packaging cells resulted in transduction of cells only within a few cell diameters of the injection tracts, lend support to this notion (Ram *et al.*, 1997; Rainov, 2000). Similar results were observed in studies involving direct intratumoral injection of defective adenovirus vectors (de Roos *et al.*, 2000; Kurihara *et al.*, 2000).

In contrast, the present study suggests that the capacity of replication-competent vectors for intercellular spread, which allows transduction of cells not initially exposed to the vector inoculum, largely circumvents such physical obstacles. Furthermore, since MuLV can infect only mitotically active cells, and the half-life of virion particles (5–8 hr) (Morgan *et al.*, 1995; Chuck *et al.*, 1996) is much shorter than the average cell cycle time of most human tumors (3–6 days), continuous release of replicating vector from initially infected cells also increases the likelihood that additional target cells will undergo division and thereby become infected.

The replication competence of the vector described here obviously raises questions about possible pathogenic effects resulting from spread of the vector in the host. Moloney MuLV is known to induce thymic lymphoma in newborn mice, and many other murine retroviruses are associated with characteristic malignancies. However, most of these viruses are not pathogenic in adult mice (Rosenberg and Jolicoeur, 1997). Nonpathogenic strains of MuLV have also been described, and these may be amenable for use in the construction of replicating vectors similar to those described here. Initial studies of the activity of amphotropic MuLV in rhesus monkeys could find no evidence of pathology in infected animals over a 3-year observation period, despite severe immune suppression at the time of infection and the administration of high doses (mean, 7.2×10^7 PFU) of replication-competent MuLV (Cornetta *et al.*, 1990, 1991b). A later study, however, revealed that MuLV can be oncogenic in primates under certain conditions, on the

basis of the observation that 3 of 10 rhesus monkey recipients of bone marrow cells infected with replication-competent MuLV developed T cell lymphoma (Donahue *et al.*, 1992; Anderson *et al.*, 1993). These results suggest that while MuLV has oncogenic potential in primates, the presence of a normally functioning immune system is sufficient to prevent the realization of this potential. As the mice used in the present study were athymic, the influence of a fully functional immune system on the spread of ZAPd-GFP has yet to be determined. The tumor microenvironment itself is known to be inherently immunosuppressive; therefore it is possible that RCR vector spread would be facilitated within the tumor even in immunocompetent hosts, while extratumoral spread would be restricted by the immune system.

The RCR vector was observed to transduce host-derived cells within the tumors, including fibroblasts and endothelial cells, apparently as efficiently as the tumor cells themselves. In some situations, for example, for angiogenesis inhibition strategies, achieving gene delivery to the nontumor cell compartment of tumors may be advantageous. These cells are more homogeneous in nature and lack the propensity of tumor cells to mutate rapidly; hence, such cells may be less likely to develop resistance to introduced therapeutic genes.

Nevertheless, the desirability of a mechanism to control RCR vector spread is underscored by the above observation that the vector efficiently transduced host-derived cells within the tumors. This was not unexpected, since Moloney MuLV is tropic for murine cells, and in its present form the vector employed in these studies is untargeted, except for its natural selectivity for proliferating cells. However, the finding that spread of this untargeted vector appeared to be confined to the tumor tissue was somewhat unexpected. Since the assay used to detect spread of the vector outside of the tumors, however, was based on amplification of the GFP transgene, the possibility remains that deletion variants of the vector lacking the transgene sequence were present in the tissues examined. Such deletion variants were not apparent by Southern blot analysis of DNA from the tumor tissue itself. The lack of detectable levels of extratumoral spread may be a consequence of the dilution of any vector that left the tumor, combined with the relative inability of MuLV to infect nonproliferating normal tissues.

As mentioned above, incorporating a suicide gene into RCR vectors would not only provide the means to kill transduced tumor cells, but would also serve as a self-limiting safety mechanism to terminate vector spread after adequate levels of transduction are achieved. An additional means of targeting spread of a replicating vector, and thereby minimizing risk to recipients, would be to engineer the virus to replicate only within particular cell subpopulations. One strategy that has been used to direct retroviral vector tropism is through the modification of the viral envelope protein to target the entry of vectors into cells expressing specific cell-surface proteins (Russell *et al.*, 1993; Kasahara *et al.*, 1994; Valsesia-Wittmann *et al.*, 1994; Snitkovsky and Young, 1998). Tight control of retroviral tropism has also been achieved by replacement of the retroviral promoter/enhancer with cell type-specific transcriptional control elements, to target expression to particular tissues (Diaz *et al.*, 1998; Jager *et al.*, 1999). The development of tissue-targeted RCR vectors would represent a significant improvement in vector technology for gene therapy of cancer.

ACKNOWLEDGMENTS

We thank Paula Cannon for critical review of the manuscript, and Peng-Xuan Liu and Erlinda M. Gordon for technical assistance. This work was supported by GTI/Novartis (Gaithersburg, MD), the Department of Defense Breast Cancer Research Program grant BC980554, and NIH grant R01 CA85908. C.R.L. is the recipient of a Breast Cancer Research Project Fellowship through the Norris Comprehensive Cancer Center, and C.-K.T. is the recipient of a Susan G. Komen Breast Cancer Foundation predoctoral fellowship.

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Received for publication October 25, 2000; accepted after revision March 5, 2001.

HUMAN GENE THERAPY 14:789–802 (May 20, 2003)

Antibody-Mediated Targeting of Replication-Competent Retroviral Vectors

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ABSTRACT

Replication-competent murine leukemia virus (MLV) vectors can be engineered to achieve high efficiency gene transfer to solid tumors *in vivo* and tumor-restricted replication, however their safety can be further enhanced by redirecting tropism of the virus envelope. We have therefore tested the targeting capability and replicative stability of ecotropic and amphotropic replication-competent retrovirus (RCR) vectors containing two tandem repeats from the immunoglobulin G-binding domain of Staphylococcal protein A inserted into the proline-rich "hinge" region of the envelope, which enables modular use of antibodies of various specificities for vector targeting. The modified envelopes were efficiently expressed and incorporated into virions, were capable of capturing monoclonal anti-HER2 antibodies, and mediated efficient binding of the virus-antibody complex to HER2-positive target cells. While infectivity was markedly reduced by pseudotyping with targeted envelopes alone, coexpression of wild-type envelope rescued efficient cellular entry. Both ecotropic and amphotropic RCR vector/anti-HER2 antibody complexes achieved significant enhancement of transduction on murine target cells overexpressing HER2, which could be competed by preincubation with excess free antibodies. Interestingly, HER2-expressing human breast cancer cells did not show enhancement of transduction despite efficient antibody-mediated cell surface binding, suggesting that target cell-specific parameters markedly affect the efficiency of post-binding entry processes. Serial replication of targeted vectors resulted in selection of Z domain deletion variants, but reduction of the overall size of the vector genome enhanced its stability. Application of antibody-mediated targeting to the initial localization of replication-competent virus vectors to tumor sites will thus require optimized target selection and vector design.

OVERVIEW SUMMARY

Nondefective retroviral vectors containing the protein A immunoglobulin G-binding domain inserted within the envelope were tested for anti-HER2 antibody-targeted transduction of breast cancer cells. Virus-antibody complexes showed efficient and specific binding to human breast cancer cells which overexpress HER2 and murine fibroblasts modified to overexpress HER2. With coexpression of wild-type envelope, antibody-mediated enhancement of infectivity on the order of 10-fold was achieved on the murine cells but not any of the human breast cancer cells. These results demonstrate that retroviral vectors can, in principle, be targeted using this approach, but also suggest that cellular determinants may play a significant role in affecting the efficiency of targeted transduction, and should be considered in the design and testing of viral targeting strategies.

INTRODUCTION

THE INABILITY OF conventional replication-defective murine leukemia virus (MLV)-based retroviral vectors to achieve effective transduction of tumors *in vivo* has been a major obstacle to gene therapy for cancer. In contrast, we have recently demonstrated that use of replication-competent retrovirus (RCR) vectors can achieve highly efficient replicative spread coupled with transmission of inserted transgenes *in vivo* in solid

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tumors, as each tumor cell that is successfully transduced then itself becomes a virus-producing cell, resulting in further transduction events even after the initial administration (Logg *et al.*, 2001b). While the use of such RCR vectors has hitherto rarely been contemplated because of the potential risks associated with uncontrolled virus spread, MLV-based RCR vectors offer significant advantages over other replication-competent viruses that are currently in development as oncolytic agents.

First, the simple RNA genome and well-characterized life cycle of MLV make it much easier to manipulate and to regulate transcription and replication of the virus with high fidelity. Second, MLV may not elicit long-term persistent inflammatory responses or cytotoxicity as has previously been reported in the case of adenovirus (Wilson, 1996) and herpes virus (Boivin *et al.*, 2002). Furthermore, the initial rationale for use of retroviral vectors in cancer gene therapy still holds true for RCR vectors, namely the lack of nuclear localization signals in their nucleocapsid complex, MLV-based vectors can only transduce cells that are actively dividing (Miller *et al.*, 1990). In fact, our previous studies have confirmed that after intratumoral injection of RCR vectors, viral replication appears to be well confined to the tumor itself without detectable spread to normal tissues (Logg *et al.*, 2001b). In addition, incorporation of a suicide gene into the RCR vector would itself constitute a built-in safeguard, because even noncancerous cells infected by the virus would eventually be eliminated by treatment, and the spread of the vector would be inherently self-limited. These intrinsic safety considerations notwithstanding, it would still clearly be advantageous to incorporate additional mechanisms so that RCR vectors would more selectively target cancer cells, thereby enhancing the safety of this vector system while enabling more efficient transduction of tumors.

To develop retroviral vectors exhibiting cell-type–specific gene transfer capability, several groups have modified the envelope protein by insertion of single-chain antibodies (scFv) (Russell *et al.*, 1993; Chu and Dornburg, 1995; Somia *et al.*, 1995; Chu and Dornburg, 1997) and peptide ligands (Kasahara *et al.*, 1994; Valsesia-Wittmann *et al.*, 1994). Retrovirus vectors packaged with chimeric scFv- or ligand-envelope proteins do show retargeting of binding tropism, albeit at the cost of losing several orders of magnitude in transduction efficiency. It has also recently been shown that targeted entry of defective retroviral vectors can be achieved more efficiently by complexing the vectors with cell-specific monoclonal antibodies (mAb), through the use of chimeric retroviral envelopes (Ohno and Meruelo, 1997) or Sindbis viral envelopes (Ohno *et al.*, 1997; Morizono *et al.*, 2001) that contain the immunoglobulin (Ig) G-binding domain of protein A derived from *Staphylococcus aureus* (Nilsson *et al.*, 1987), which has a strong affinity for the Fc region of various mammalian IgGs.

We therefore sought to apply this modular strategy for targeting specific cell types via complexed antibodies to RCR vectors. To this end, we have introduced an insert containing 2 tandem repeats of the Staphylococcal protein A IgG-binding domain (Z domain) into the proline-rich region (PRR) of ecotropic and amphotropic RCR vector envelopes. In order to target vectors displaying the Z domain, we used an mAb directed against the human epidermal growth factor-like receptor-2 (HER2)/neu oncogene product, which belongs to the family of epidermal growth factor receptors and is overexpressed in approximately 30% of all human breast cancer cells. This anti-HER2 mAb (Park *et al.*, 1999) has previously been demonstrated to specifically bind the extracellular domain of HER2 with high affinity and to trigger endocytosis of the antibody-HER2 complex in HER2-overexpressing breast cancer cells. In these studies, we examined the targeting efficiency of Z domain/anti-HER2 antibody-targeted retrovirus vectors in transducing both a murine target cell line as well as human breast cancer cell lines overexpressing HER2. As any reduction of replicative efficiency selects for rapid loss of disadvantageous sequences from replicating viruses, we also examined the stability of the introduced Z domain sequences in the context of RCR vectors over multiple serial infection cycles.

MATERIALS AND METHODS

Cell lines

293T (DuBridge *et al.*, 1987), NIH 3T3 (Jainchill *et al.*, 1969), and NIH/189 (NIH 3T3 cells modified to stably express HER2) (Di Fiore *et al.*, 1987), were cultivated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Human breast cancer cells SK-BR-3, BT-474, and MDA-MB-453, which overexpress HER2, and MDA-MB-231, which express low levels of HER2, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell lines were cultured according to ATCC instruction.

Retroviral vector plasmid construction

Plasmid constructs are indicated by the letter "p" (e.g., pZV-GFP) to distinguish them from the virus derived from the plasmid construct (e.g., ZV-GFP). Plasmid pZAPd-GFP (Logg *et al.*, 2001a,b), also named as pZE-GFP, is a replication-competent ecotropic murine leukemia virus (MLV) vector containing internal ribosome entry site (IRES)-GFP cassette positioned between the *env* gene and 3′ untranslated region (UTR). pZ/Cee-S is an expression plasmid for the ecotropic envelope that PRR has been replaced with amphotropic envelope PRR containing two tandem repeats of the IgG-binding domain (Z domain) of protein A. The envelope region of pZE-GFP was excised with *Hpa*I and replaced by the corresponding fragment excised with *Hpa*I from pZ/Cee-S, producing pZE-ZZ-GFP. The N terminus of the ecotropic envelope surface subunit (SU) of pZE-ZZ-GFP was replaced with N terminus of 4070A amphotropic envelope SU, generating plasmid pAZE-ZZ-GFP. Plasmid pAZE-GFP is derived from pZE-GFP in which the ecotropic envelope was replaced with the 4070A amphotropic envelope.

We also introduced a synthetic 163-bp vascular endothelial growth factor (VEGF) IRES (Stein *et al.*, 1998) followed by a polylinker into an ecotropic MLV vector pZAP2 (Logg *et al.*, 2001b) between the *env* gene and the 3′ UTR. This plasmid was designated pZV. We then inserted into the polylinker a GFP cDNA from pGFPemd-cmv [R] control vector (PerkinElmer Life Sciences, Boston, MA), and the resulting construct was named pZV-GFP. A fragment composed of the VEGF IRES and GFP was then excised from pZV-GFP and cloned into pZE-ZZ-GFP, producing pZV-ZZ-GFP.

Fluorescence-activated cell sorting (FACS) analysis

Cells were washed with phosphate-buffered saline (PBS), trypsinized, or detached with 10 m*M* ethylenediaminetetraacetic acid (EDTA) and collected by low-speed centrifugation. Cells were resuspended in PBS at approximately 5×10^5 cells per milliliter and analyzed for fluorescence with a Becton Dickinson FACScan using a fluorescein isothiocyanate filter set.

Retroviral vector production and titer determination

Viral particles were produced by transient transfection of the virus-encoding plasmids into 293T cells using calcium phosphate precipitation as described previously (Pear *et al.*, 1993). The virus-containing supernatant was collected 48 hr post-transfection, filtered through a 0.45-μm filter and used immediately or frozen for later use.

For titer determination, viral supernatant was complexed with or without anti-HER2 mAb at 4°C for 1 hr, then filtered through a 300-kd molecular weight cutoff filter by low-speed centrifugation to remove excess unbound mAb. The unfiltered supernatant was then added to approximately 20% confluent NIH 3T3 or NIH/189 or 30% confluent SK-BR-3, BT-474, MDA-MB-453 or MDA-MB-231 in 6-well plates. Viral supernatant was then removed 24 hr postinfection, and cells were incubated in regular media with 50 μM 3′-azido-3′-deoxythymidine (AZT) (Sigma, St. Louis, MO) for 24 hr (NIH 3T3 and NIH/189) or 72 hr (SK-BR-3, BT-474, MDA-MB-453 and MDA-MB-231), and subjected to FACS. The viral titer was calculated according to the formula: transducing units (TU)/ml = number of cells counted immediately prior to infection × percentage of transduced cells reported from FACS analysis/volume (ml) of viral supernatant.

Virus-cell binding assay

Cells to be tested for virus binding were collected by nonenzymatic treatment with 10 m*M* EDTA, and distributed to polypropylene tubes (2×10^5 cells per sample). Viral supernatant was complexed with or without anti-HER2 mAb (Park *et al.*, 1999) for 40 min, then filtered through a 300-kd molecular weight cutoff filter by low-speed centrifugation to remove excess unbound mAb and soluble envelope proteins. The unfiltered supernatant was then added to each tube containing cells described above and incubated at 37°C for 20 min. After incubation with virus, cells were washed twice with 2 ml of cold wash buffer (5% goat serum in PBS) and then complexed with 100 μl of the rat monoclonal anti-SU antibody 83A25 (generously provided by Dr. Leonard Evans, Rocky Mountain Labs, Hamilton, MT) (Evans *et al.*, 1990) at 4°C for 40 min. They were washed again with 2 ml of cold wash buffer and then incubated with 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rat IgG (CalTag, Burlingame, CA) at 4°C in the dark for 40 min. After washing, the cells were resuspended in cold PBS and subjected to FACS analysis.

Immunoblot assay

Viral supernatants from transfected 293T cells were harvested and pelleted through 20% sucrose at 15,000*g* and 4°C for 1 hr. Viral pellets were resuspended in sodium dodecyl sulfate (SDS) gel-loading buffer (62.5 m*M* Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 100 m*M* DTT, 0.01% bromophenol blue) and boiled for 5 min. Viral protein samples were run on SDS-7.5% or 10% polyacrylamide gels and transferred onto polyvinyl difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The blots were blocked for 1 hr at room temperature in TBST (100 m*M* Tris-HCl [pH 7.5], 150 m*M* NaCl, 0.1% Tween 20) with 5% nonfat dry milk (Bio-Rad), and incubated overnight with goat anti-MLV IgG (lot# 71S000126; Microbiological Associates, Inc., Rockville, MD) at 1:1,000 dilution at 4°C. After being washed in TBST for 5 min for 3 times, the blots were incubated with horseradish peroxidase (HRP)-conjugated swine anti-goat IgG (CalTag) at 1:3,000 dilution at room temperature for 1 hr and then washed in TBST for 5 min for 3 times. Specific protein signals were detected with an enhanced chemiluminescence (ECL) kit from Amersham.

Coprecipitation assay

Viruses were precomplexed with mouse anti-HER2 mAb at room temperature for 1 hr and pelleted through 35% sucrose cushion at 15,000*g* and 4°C for 1 hr. Pelleted viral complexes were subjected to Western analysis with HRP-conjugated goat anti-mouse IgG (CalTag).

Competition assay

NIH/189 cells (5×10^5) in 6-well plates were preincubated with or without excessive amount of mouse anti-HER2 antibody (50 μg) at 37°C for 1.5 hr. After cells were washed with PBS and fresh culture media was added, viral supernatants (ZV-ZZ-GFP plus ecotropic env) precomplexed with or without anti-HER2 antibody were added to such cells. Viral transduction was evaluated by FACS analysis after 2 days.

Serial transduction with replicating vector

NIH 3T3 cells at 20% confluence were infected with virus stock at a multiplicity of infection (MOI) of 0.05 in the presence of 3 μg/ml polybrene (Sigma). At 2 days postinfection, the cells were split and at 4 days postinfection, the culture supernatant was diluted 30-fold and used to infect a fresh population of NIH 3T3 cells. This cycle was repeated several additional times.

Polymerase chain reaction analysis of viral deletions

Unintegrated proviral DNA was isolated from each serially infected NIH 3T3 population by a modified Hirt procedure (Joseph, 1981). Briefly, on the fourth day after each serial infection, infected cells were cocultivated with uninfected NIH 3T3 cells at a ratio of 1:3, respectively. Three days later, extrachromosomal DNA was isolated by lysis of the cells with SDS followed by salt precipitation of protein and chromosomal DNA and phenol-chloroform extraction of the resulting supernatant. Hirt DNA was used as the template for polymerase chain reaction (PCR) amplification of the envelope using the following primers flanking the PRR sequence: 5′-CTCACCTCT-GACCAGGCTGTC-3′ and 5′-CTGCTCCTATGCAGAGTC-CCT-3′. Briefly, amplification was performed in a reaction volume of 50 μl under the following conditions: 10 μl sample Hirt DNA, 0.2 μM dNTPs, 0.4 μM of each primer, 1× PCR buffer, and 5 units *Taq* polymerase (Invitrogen, Carlsbad, CA).

Products were amplified by 30 cycles of successive incubation at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min 30 sec, respectively. Amplification cycles were then followed by 10-min extension at 72°C.

RESULTS

Construction and production of RCR vectors displaying IgG-binding domains of protein A

We have previously described replication-competent MLV-based retrovirus vectors that contain an internal ribosome entry site (IRES)-transgene expression cassette inserted precisely at the boundary between the *env* gene and the 3′ UTR (Logg *et al.*, 2001a). These vectors, here designated ZAPd-GFP (ZE-GFP) and AZE-GFP, encode the ecotropic and amphotropic MLV envelope proteins, respectively. The ecotropic MLV envelope cannot mediate infection of human cells because the sequence of its receptor, the murine cationic amino acid transporter mCAT-1 (Albritton *et al.*, 1989), is divergent between murine and non-murine species. In contrast, the receptor for the amphotropic 4070A MLV envelope, the phosphate transporter PiT-2 (Miller *et al.*, 1994; van Zeijl *et al.*, 1994) is highly conserved, hence amphotropic MLV can infect the cells of many species.

To modify the envelope of these RCR vectors to allow antibody-mediated targeting, we introduced two tandem repeats of the Z domain of protein A into the proline-rich region (PRR) of the amphotropic MLV envelope, and this modified PRR sequence was used to replace the corresponding PRR regions of ZE-GFP and AZE-GFP, now designated ZE-ZZ-GFP and AZE-ZZ-GFP, respectively (Fig. 1A). Insertion of modified amphotropic PRR domains has previously been shown not to impede the function of the envelope in mediating cell entry of replication-defective vectors via the normal virus receptor (Weimin Wu *et al.*, 1998). The orientation and integrity of the Z domains in pZE-ZZ-GFP and pAZE-ZZ-GFP were confirmed by sequencing.

We have previously established that these RCR vectors can stably retain their inserted IRES-transgene cassettes at the *env*-3′ UTR boundary for multiple infection cycles, as long as the size of the inserted sequences is on the order of 1.3 kb (and hence the total size of the genomic mRNA is approximately 9.6 kb) (Logg *et al.*, 2001a). However, the introduction of exogenous sequences into the envelope itself in order to modify the binding tropism of the virion would further increase the total size of the RCR vector genome. Therefore, to reduce the overall size of RCR vectors and thereby enhance their genomic stability even with additional insertions into the *env* gene, we also constructed new RCR vectors containing shorter IRES sequences. In place of the relatively long IRES sequence we had previously used, which was derived from encephalomyocarditis virus (EMCV) and is approximately 550 bp in length, a sequence containing a synthetic 163-bp IRES derived from the vascular endothelial growth factor (VEGF) gene (Stein *et al.*, 1998) followed by the green fluorescent protein (GFP) transgene was similarly introduced into the ecotropic MLV-based RCR vector between the *env* gene and the 3′ UTR, and the resulting construct was designated pZV-GFP (Fig. 1A). The fragment containing the VEGF IRES-GFP cassette was also used to replace the EMCV IRES-GFP cassette in the targeted RCR construct pZE-ZZ-GFP, producing pZV-ZZ-GFP (Fig. 1A).

To examine whether the RCR vectors could express the GFP transgene, we transfected each construct into 293T cells. At 2 days posttransfection, 293T cells transfected with these plasmids exhibited green fluorescence when observed microscopically under UV transillumination, revealing that both EMCV and VEGF IRES sequences could mediate expression of the GFP transgene (data not shown), and the supernatant culture medium from each transfection was harvested and filtered to remove cell debris.

Incorporation of chimeric envelopes into RCR vector particles

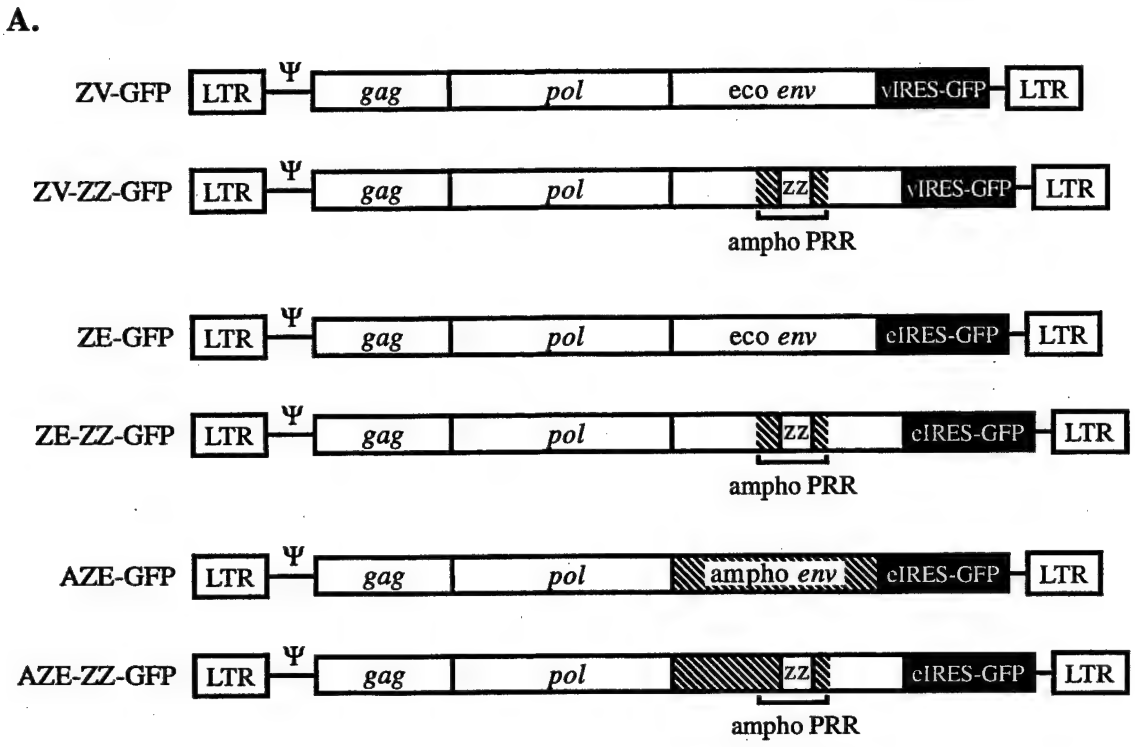
To determine whether Z domain-containing envelopes can be successfully incorporated into viral particles, immunoblot analysis using a polyclonal anti-MLV antibody was performed on the viral supernatants harvested for each construct.

The results indicate that Z domain-containing envelopes can be expressed and incorporated into virions at levels comparable to those of wild-type envelope. The parental ecotropic vectors ZV-GFP and ZE-GFP showed the expected M_r 70-kd band corresponding to the wild-type ecotropic envelope SU protein gp70, and similarly the parental amphotropic AZE-GFP showed a cross-reacting M_r 70-kd band corresponding to the amphotropic SU protein. In contrast, all constructs containing the Z domain insertion showed a positive signal around M_r 85 kd, which is the estimated molecular mass of the Z domain-containing envelope proteins (Fig. 1B). The signal intensities of these higher molecular weight species were comparable to those observed for the wild-type envelopes, indicating that envelope protein incorporation was not significantly affected by the Z domain insertion.

To evaluate the functional integrity of the Z domain within the context of the envelope, we also performed coprecipitation assays in which the viral particles were complexed with a mouse anti-HER2 mAb (Park *et al.*, 1999) and pelleted through a sucrose cushion to remove any excess unbound antibodies. Pelleted viral complexes were subjected to Western blot analysis to detect mouse immunoglobulins using an anti-mouse IgG antibody. A signal corresponding to the captured anti-HER2 mAb was observed to be present in the viral complexes with Z domain-containing envelopes, but not in those coated with only wild-type envelope (data not shown), indicating a physical interaction between the Z domain and mAb.

Enhanced binding of Z domain/HER2 antibody-targeted RCR vectors to HER2 overexpressing cells

To assess whether the RCR vectors, ZV-ZZ-GFP and AZE-ZZ-GFP, can specifically bind HER2 overexpressing cells when complexed with an anti-HER2 antibody, we conducted virus-cell binding assays in the presence or absence of the antibody. For these binding assays, NIH/189, a NIH 3T3-derived murine fibroblast cell line stably expressing HER2, and the HER2-overexpressing human mammary carcinoma cell line SK-BR-3 were used as target cells, and the parental NIH 3T3 cells were used as negative control cells. Antibody-complexed or uncomplexed virion preparations were incubated with each cell population at



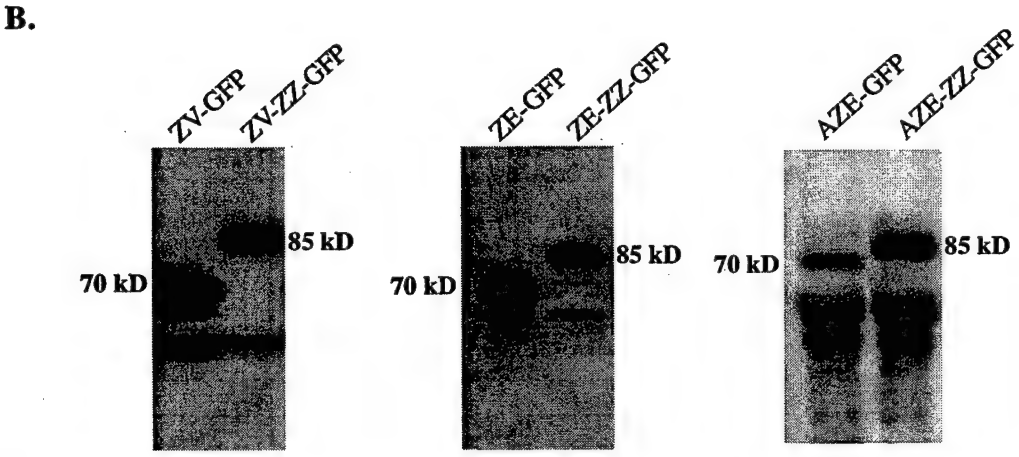


FIG. 1. **A**: Schematic representation of replication-competent retroviral vectors. ZV-ZZ-GFP, ZE-ZZ-GFP, and AZE-ZZ-GFP contain two tandem repeats of immunoglobulin G-binding (Z) domain of protein A within the *env*. vIRES: VEGF IRES. eIRES: EMCV IRES. **B**: Western blot analysis of retroviral envelope protein. Virus supernatants from transfected 293T cells were harvested, filtered, concentrated, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting with goat anti-murine leukemia virus (MLV) IgG and horseradish peroxidase (HRP)-conjugated swine anti-goat IgG. The 70-kd bands represent the wild-type envelope SU protein gp70 and the 85-kd bands correspond to the Z domain-containing envelope proteins.

4°C to prevent endocytosis, excess unbound virus was washed away, and the bound virus was detected by FACS analysis using an antibody directed against the C-terminal portion of MLV *env* SU, an epitope that is still retained within the modified envelope proteins. In this assay, the magnitude of the shift in fluorescence intensity correlates with the amount of bound virus on the cell surface (Kadan *et al.*, 1992).

On parental NIH 3T3 cells that do not express HER2, a small but consistent shift in fluorescence intensity was observed for both ZV-ZZ-GFP and AZE-ZZ-GFP in the absence of the anti-HER2 antibody (Fig. 2, hatched histograms in panels A and D), and this shift was not enhanced by pre-complexing the antibody to the virions (Fig. 2, black histograms in panels A and D). Virus-cell binding was also detected in the absence of anti-HER2 antibody for ecotropic ZV-ZZ-GFP on murine NIH/189 cells and for amphotropic AZE-ZZ-GFP on both NIH/189 cells

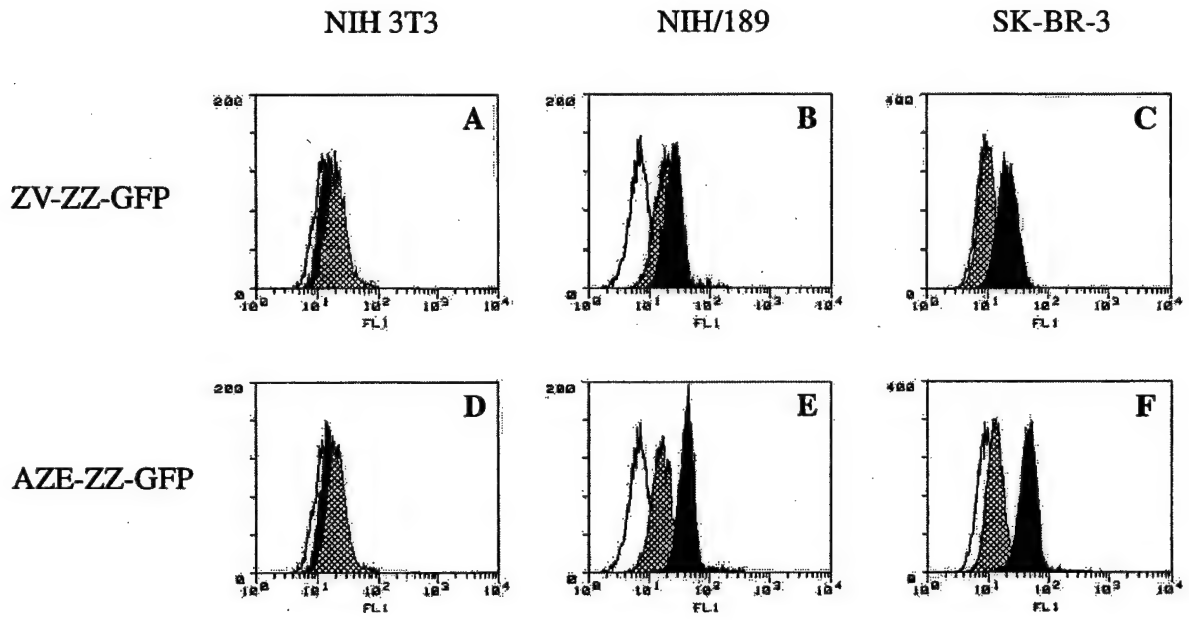


FIG. 2. Binding assays of Z domain-bearing murine leukemia virus (MLV) vectors. Binding assays were performed with virus supernatants of 293T cells transfected with the indicated vectors. White histogram: no virus. Hatched histogram: indicated virus without the supplement of anti-HER2 antibody. Black histogram: indicated virus with the supplement of anti-HER2 antibody. Y-axis: cell number. X-axis: fluorescence intensity.

as well as human SK-BR-3 cells, presumably due to the presence of the intact receptor binding domain in the envelope of these vectors (Fig. 2BEF, hatched histograms). As expected, in the absence of antibody, ZV-ZZ-GFP showed no significant shift in target cell fluorescence (Fig. 2C, hatched histogram) because human cells have no mCAT-1 receptor for ecotropic MLV binding.

In contrast, strong enhancement of both ZV-ZZ-GFP and AZE-ZZ-GFP binding was consistently observed in the presence of anti-HER2 antibody on the HER2-expressing target cells NIH/189 (Fig. 2B and 2E, black histograms) as well as SK-BR-3 (Fig. 2C and 2F, black histograms), compared to virus binding in the absence of antibody (Fig. 2B and 2C and 2E and 2F, hatched histograms). Thus the display of anti-HER2 mAb tethered to Z domains in the PRR of MLV envelopes enhanced binding of the viral particles to HER2 antigen on the target cell surface. It is notable that the ecotropic ZV-ZZ-GFP when conjugated with anti-HER2 mAb was able to bind to the human breast cancer cell SK-BR-3 (Fig. 2C, black histogram), suggesting cross-species retargeting of MLV vector binding tropism purely via the mAb.

Antibody-enhanced transduction of RCR vectors

We then sought to determine whether the observed HER2-specific antibody-mediated enhancement in target cell binding by ZV-ZZ-GFP and AZE-ZZ-GFP could mediate a concomitant enhancement in their ability to transduce the HER2-positive murine NIH/189 and human SK-BR-3 cell lines. Additional human mammary carcinoma cell lines BT-474 and MDA-MB-453, which both overexpress HER2, as well as MDA-MB-231, which expresses only basal levels of HER2, were also tested for comparison. As tropism-modified envelopes frequently exhibit impairment of post-binding fusion activation, we also performed parallel experiments in which wild-type *env* was co-expressed along with the Z domain envelopes.

Target cell transduction was assessed by FACS analysis for GFP expression 48 to 96 hr after inoculation of recombinant viruses complexed with anti-HER2 antibodies, compared to uncomplexed viruses. As shown in Figure 3A, an approximately 10-fold enhancement of NIH/189 target cell transduction was observed with ZV-ZZ-GFP when the Z domain-containing envelope was co-expressed with wild-type ecotropic envelope in the presence of precomplexed anti-HER2 antibodies, although this was not observed when ZV-ZZ-GFP was pseudotyped with the Z domain-containing envelope alone. In contrast, the parental control ZV-GFP virus, showed good overall transduction titers as expected (Table 1), but showed no difference in transduction efficiency in the presence or absence of antibodies (Fig. 3A), presumably because without the Z domain, the virus failed to form complexes with the antibody.

Similarly, AZE-ZZ-GFP coexpressed with wild-type amphotropic envelope exhibited enhanced transduction of NIH/189 cells when complexed with anti-HER2 antibodies (Fig. 4A), whereas the parental AZE-GFP vector showed no specificity in the presence or absence of antibodies despite good overall transduction levels on NIH/189 cells. Interestingly, transduction by AZE-ZZ-GFP pseudotyped with the Z domain envelope alone was markedly diminished upon complex formation with the antibodies (Fig. 4A), a phenomenon that was not observed with ZV-ZZ-GFP (Fig. 3A). In stark contrast to the NIH/189 target cells, neither SK-BR-3, BT-474, MDA-MB-453, nor MDA-MB-231 can

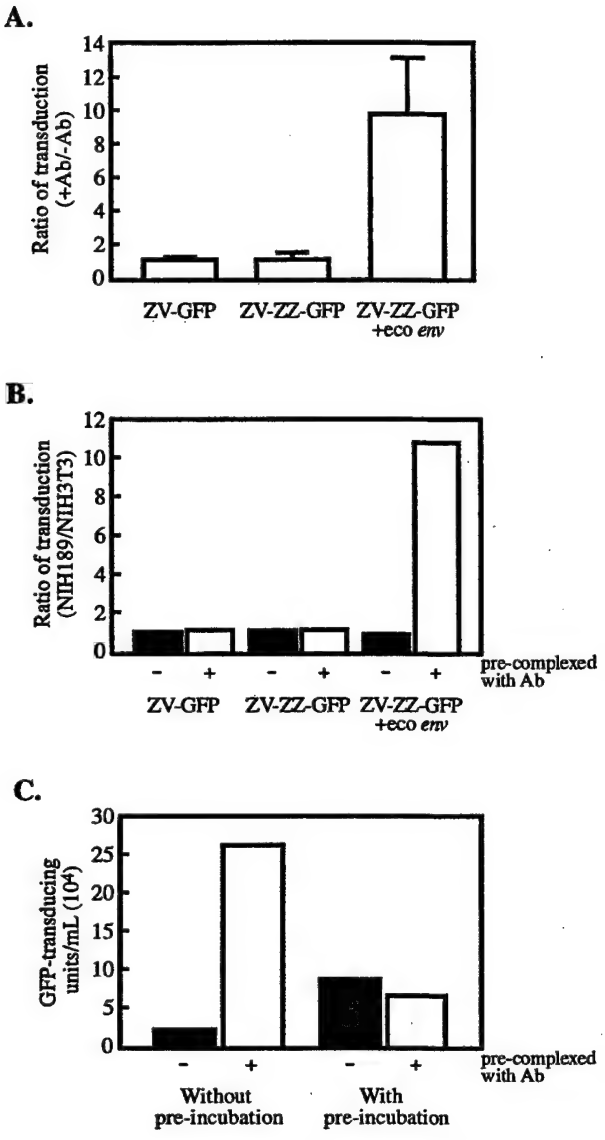


FIG. 3. Antibody-mediated targeting of ecotropic murine leukemia virus (MLV) vectors to HER2-overexpressing cells. **A**: Antibody-enhanced transduction of recombinant retrovirus. Virus supernatants derived from transfected 293T cells were precomplexed with or without anti-HER2 antibodies and added to NIH/189 in 6-well plates. Viral transduction was evaluated by fluorescence-activated cell sorter (FACS) analysis after 2 days. Shown values were obtained from five independent experiments, and error bars represent standard deviations. **B**: Cell-specific targeting of recombinant retrovirus. Virus supernatants were precomplexed with or without anti-HER2 antibodies and added to NIH/189 and NIH 3T3 cells in 6-well plates. Viral transduction was evaluated by FACS analysis after 2 days. **C**: Competitive inhibition of viral transduction by the blocking of cell surface HER2. NIH/189 cells were preincubated with or without excessive amount of anti-HER2 antibody. Viral supernatants (ZV-ZZ-GFP plus ecotropic *env*) precomplexed with or without anti-HER2 antibody were added to such cells in 6-well plate. Viral transduction was evaluated by FACS analysis after 2 days. Titers are on the order 1×10^4.

be specifically targeted via the AZE-ZZ-GFP plus amphotropic *env*/anti-HER2 antibody complexes (Fig. 4B).

Cell specificity of antibody-targeted replication-competent retrovirus vectors

We then assessed whether the enhanced level of transduction efficiency due to anti-HER2 antibody-mediated binding was specific for HER2-positive cells. No enhancement of transduction was observed on the parental NIH 3T3 cells when ZV-ZZ-GFP coexpressing wild-type ecotropic envelope was complexed with anti-HER2 antibody (Table 1), showing that the infection enhancement is highly dependent on the presence of both HER2 on the target cell surface and anti-HER2 antibody on the virion. Transduction titers of the recombinant RCR vectors complexed with anti-HER2 antibodies were approximately 10-fold higher on HER2-positive NIH/189 target cells than titers on HER2-negative parental NIH 3T3 cells (Fig. 3B).

Competitive inhibition of viral transduction by blocking of cell surface HER2

To verify directly whether the enhancement of transduction observed for ZV-ZZ-GFP coexpressing ecotropic *env* and complexed with anti-HER2 antibodies indeed involved binding to the HER2 receptor, we repeated the assays under conditions in which NIH/189 cells were preincubated with an excess of free anti-HER2 antibodies prior to infection. The quantity of excess antibody used (50 μg) represents the equivalent of 2×10^{14} molecules, and as competitive inhibition was performed by preincubation of free antibodies with 5×10^5 NIH/189 target cells prior to challenge with virus-Ab complexes, this represents a ratio of approximately 4×10^8 anti-HER2 antibody molecules per cell, which should be in considerable excess compared to the number of HER2 receptors on the surface of each target cell and therefore should be sufficient to efficiently block subsequent binding of virus-Ab complexes. As shown in Figure 3C, the ZV-ZZ-GFP+ecotropic *env*/anti-HER2 antibody complexes failed to exhibit enhanced titer on NIH/189 cells that had been preincubated with anti-HER2 mAb (Fig. 3C, open bar with preincubation vs. open bar without preincubation). Interestingly, when the recombinant virus was not precomplexed with anti-HER2 mAb, the transduction level obtained in the presence of antibody preincubation was 4-fold higher than that without antibody preincubation, suggesting that prebound antibodies might mediate some level of enhancement through interaction with the Z domain (Fig. 3C, solid bar with preincubation vs. solid bar without preincubation).

Replicative kinetics of Z domain-containing RCR vectors

To assess the genomic stability of the Z domain-containing RCR vectors over serial infection cycles in culture, we infected NIH 3T3 cells with ZE-ZZ-GFP at an initial multiplicity of infection (MOI) of 0.05. At various time points after infection, we analyzed the cells for expression of GFP by FACS (Fig. 5). An equal amount of ZE-GFP was used to infect parallel plates of NIH 3T3 cells as a positive control, and also analyzed by FACS.

TABLE 1. TITER OF RCR VECTORS

	NIH 3T3		*NIH/189*	
	−Ab	*+Ab*	*−Ab*	*+Ab*
ZV-GFP	4.7×10^5	4.3×10^5	4.6×10^5	4.7×10^5
ZV-ZZ-GFP	5.9×10^4	4.6×10^4	$6.5 \pm 1.9 \times 10^4$	$5.5 \pm 3.2 \times 10^4$
ZV-ZZ-GFP + e-env	6.3×10^4	5.1×10^4	$5.8 \pm 2.2 \times 10^4$	$5.6 \pm 3.1 \times 10^5$
AZE-GFP			$8.8 \pm 0.4 \times 10^5$	$8.2 \pm 0.9 \times 10^5$
AZE-ZZ-GFP			$2.9 \pm 0.4 \times 10^4$	$2.5 \pm 0.1 \times 10^3$
AZE-ZZ-GFP + a-env			$2.4 \pm 0.1 \times 10^4$	$7.0 \pm 0.2 \times 10^5$

Titers (transducing units per milliliter [TU/ml]) are expressed as mean ± standard deviation (SD); −Ab, without precomplexing with anti-HER2 antibody; +Ab, with precomplexing with anti-HER2 antibody; e-env, ecotropic envelope; a-env, amphotropic envelope; RCR, replication competent retrovirus.

At 2 days postinfection, GFP fluorescence was detected in only a small percentage of the cells infected by ZE-ZZ-GFP. At day 9, approximately three quarters of the population exhibited fluorescence, and by day 14, almost all cells fluoresced, demonstrating that ZE-ZZ-GFP can effect the transfer of the GFP to nearly the entire cell population (Fig. 5). However, the time course of ZE-ZZ-GFP replication appeared to be severely attenuated compared to the parental ZE-GFP virus. Similar results were obtained for AZE-ZZ-GFP compared to the wild-type AZE-GFP replication (Fig. 5).

We also tested the ability of the ZV-GFP and ZV-ZZ-GFP to replicate and spread in cell culture. In general, the VEGF IRES was observed to be approximately 5- to 10-fold weaker than the EMCV IRES in directing the translation of the second cistron, as observed by the mean fluorescence intensity of transduced cells by FACS analysis; however, the shifted cell population was clearly positive. For ZV-GFP, by day 6 nearly all of the cells expressed GFP, thus indicating that the VEGF IRES-containing RCR vector also retains transgene expression as it spreads through the cell culture. For ZV-ZZ-GFP, the expression of GFP in the culture also increases with time, although the time course is again attenuated compared to the parental ZV-GFP.

Sequence analysis of envelope region of Z domain-containing RCR vectors

To examine the stability of ZE-ZZ-GFP on replication in cell culture, we prepared Hirt DNA from each of the first 3 NIH 3T3 populations serially infected with ZE-ZZ-GFP (Fig. 6A). Hirt DNA were amplified by PCR and their products were resolved by agarose electrophoresis as shown in Fig. 6B. For the first infection cycle, a single band, approximately 170 bp shorter than the expected band, appeared (Fig. 6B). Over the next two infections, three or four bands, each smaller than the expected band, were also observed. The dominant species in the latter infection cycles was excised from the agarose gel, purified, and subsequently sequenced. The sequencing outcome shows that this deletion variant does not carry any portion of the Z domain and displays a C-terminal truncation of the wild-type amphotropic PRR (Fig. 6B). In contrast, the ZE-GFP parental control shows no such deletions over multiple rounds of infection (data not shown).

We also examined the stability of ZV-ZZ-GFP, to investigate whether the smaller size of the Z domain-containing RCR vector might contribute to increased stability. Sequence analysis of the PCR products amplified from ZV-ZZ-GFP Hirt DNA indicated that the deletion mutant envelope retained one complete

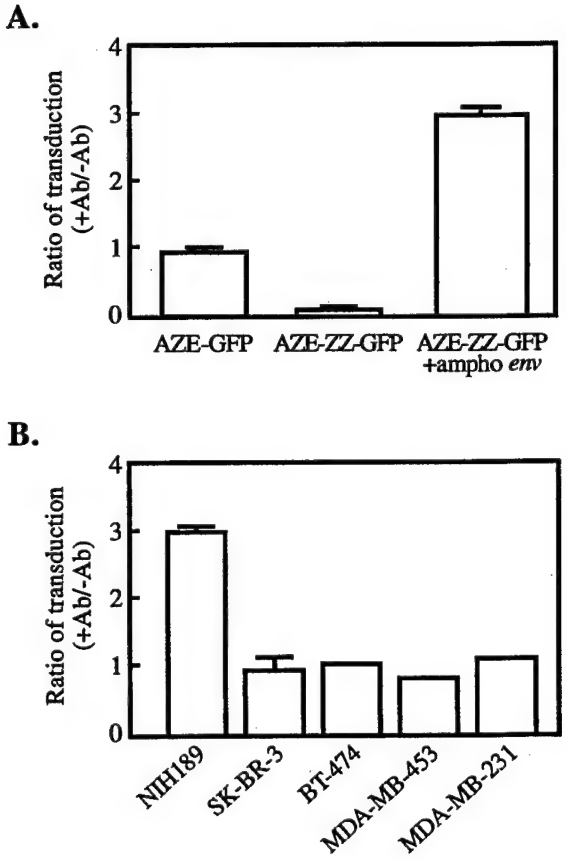


FIG. 4. Antibody-enhanced transduction of amphotropic murine leukemia virus (MLV) vectors to HER2-overexpressing cells. Virus supernatants were precomplexed with or without anti-HER2 antibodies and added to cells in 6-well plates. **A**: Viral transduction was evaluated by FACS analysis after 2 days for NIH/189. **B**: Viral transduction (AZE-ZZ-GFP plus amphotropic *env*) was evaluated by FACS analysis after 4 days for SK-BR-3, BT-474, MDA-MB-453, and MDA-MB-231.

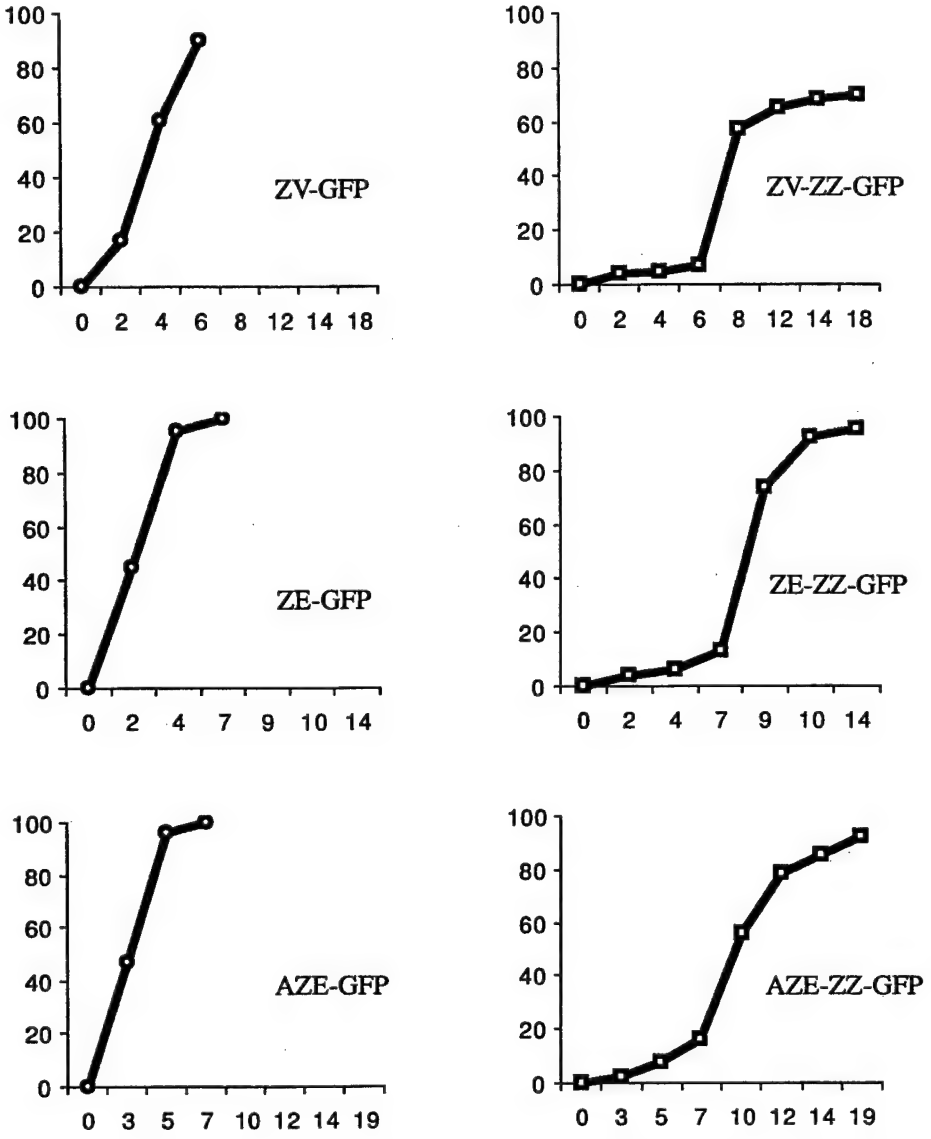


FIG. 5. Fluorescence-activated cell sorter (FACS) analysis of green fluorescent protein (GFP) expression from replication-competent retrovirus (RCR) vector spread at various time points after initial infection of NIH 3T3 cells. Y-axis: % of cells expressing GFP. X-axis: days after infection.

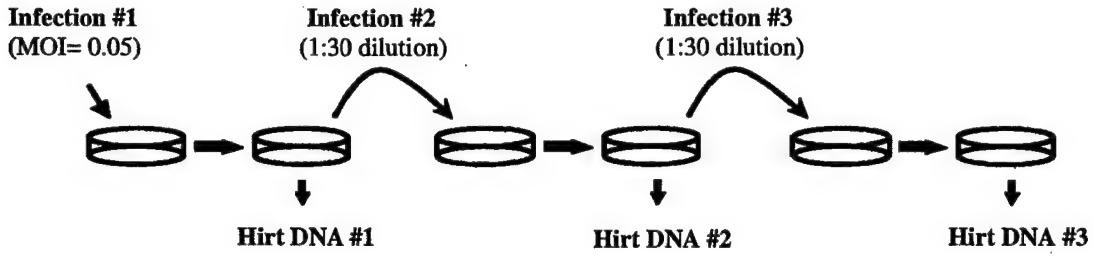
Z domain (Fig. 6C), and thus would presumably be functional for binding the Fc region of IgG, although at lower affinity.

DISCUSSION

In this study, we investigated the possibility of engineering a stable, nondefective retroviral vector for use in targeted transduction of breast cancer cells. These vectors contain the Z domain of protein A within the envelope PRR to allow conjugation of the vectors to anti-HER2 antibodies. The use of antibody-antigen interactions as the basis for targeting may have great benefit because retargeting could be achieved simply by changing the complementary mAb. However, in applying this strategy we have observed impairment of cellular entry on insertion of the Z domain and complexing with antibodies in the absence of wild-type envelope coexpression; on complementation with wild-type envelope, antibody-targeted envelopes achieved enhanced selectivity of infection for an engineered murine cell line expressing the target antigen but failed to achieve similar results using a human cell line that naturally overexpresses the target. Thus, these results suggest that cellular determinants may also play a significant role in affecting the efficiency of targeted transduction, and should be considered in the design and testing of viral targeting strategies.

The insertion site chosen to display the Z domain in retroviral vectors was the PRR, as it has previously been reported (Weimin Wu *et al.*, 1998; Kayman *et al.*, 1999; Lavillette *et al.*, 2001a) that the hypervariable C-terminal region of the PRR was not absolutely required for MLV envelope function, that

A.



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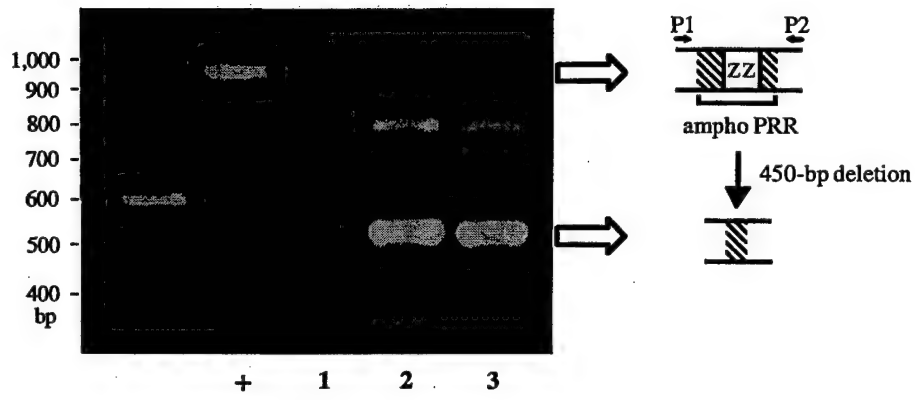

C.

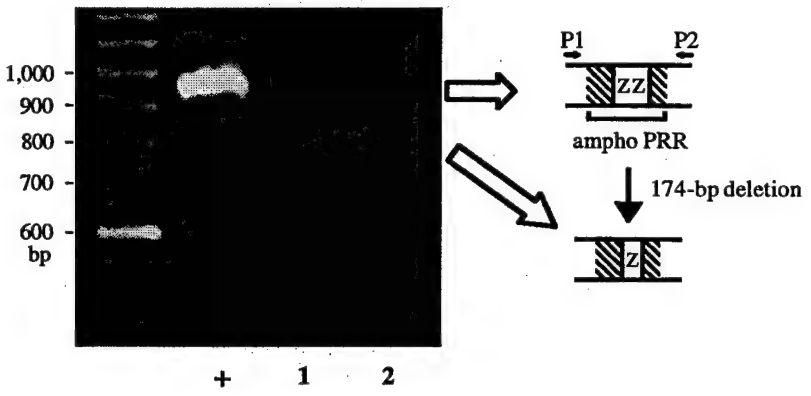

FIG. 6. Sequencing analysis of Z domain-deleted revertants. **A**: Diagram illustrating the procedure of the experiments. NIH 3T3 were infected with ZE-ZZ-GFP or ZV-ZZ-GFP at a multiplicity of infection (MOI) of 0.05. Serial infections were conducted in which a 30-fold dilution of virus supernatant from each cycle was used to infect the subsequent cell population. Each population of infected cells was used for the extraction of Hirt DNA. **B**: Deletion detection of ZE-ZZ-GFP. Each DNA template was used to generate PCR product by using primers P1 and P2. Subsequently, polymerase chain reaction (PCR) products were analyzed on a 1.5% agarose gel. Major PCR products of the second and third Hirt DNA were sequenced using the primer P1. The 450-bp deletion found within PRR is shown. +: plasmid pZE-ZZ-GFP. 1–3: first, second, and third ZE-ZZ-GFP Hirt DNA. **C**: Deletion detection of ZV-ZZ-GFP. Each DNA template was used to generate PCR product by using P1 and P2. Subsequently, PCR products were analyzed on a 1.5% agarose gel. PCR products of Hirt DNA were sequenced using the primer P1. The Z domain (174 bp) deletion found within PRR is shown. +: plasmid pZV-ZZ-GFP. 1: first ZV-ZZ-GFP Hirt DNA. 2: second ZV-ZZ-GFP Hirt DNA.

the ecotropic PRR can be replaced by the amphotropic PRR without significantly affecting virus titer, and that the amphotropic PRR can tolerate the insertion of a peptide-binding domain, making it a potentially useful site for constructing targetable retroviral vectors. We used a site within the PRR that has previously been demonstrated to tolerate insertions of 16 amino acids with only slight reduction of titers from 4×10^6 to 1.5×10^6 TU per milliliter (Weimin Wu *et al.*, 1998) and thus does not appear to greatly affect the titer of replication-defective vectors; however, more subtle effects of such insertions on impairing infectivity may be more apparent with larger insert sizes, or as a result of steric hindrance on complex formation with the targeting antibodies. Certainly, our present results indicate that titers are reduced by at least 10-fold on insertion of the 340-bp Z domain tandem repeats in the same insertion site.

Murine NIH 3T3 cells overexpressing HER2 were infected by the ZV-ZZ-GFP and AZE-ZZ-GFP in the presence or absence of anti-HER2 antibody. We found that the infection was markedly enhanced in the presence of antibody, but only when wild-type envelope was coexpressed on the vector surface along with the targeted envelope. These results indicate that the modified envelope can mediate efficient anchorage of vector to the cells through the anti-HER2 antibody, but the ability of the targeted envelope itself to mediate virus-cell membrane fusion and vector entry into the cells appears to be greatly attenuated. Several reports suggest that the proline-rich domain is not just a flexible linker but rather a functional domain; it was found (1), in some cases, to influence receptor recognition; (2) to be important for stabilization of SU-transmembrane protein (TM) interaction, and (3) to affect virus fusogenicity possibly by altering glycosylation. A direct role of the natural proline-rich region of the SU in stabilization of the conformation and fusogenicity of the MLV envelope was recently demonstrated (Valsesia-Wittmann, 2001). In the present study, both ecotropic and amphotropic envelopes containing the ZZ insertion showed significant binding to target cells in the absence of anti-HER2 antibody, suggesting that the overall conformation of the receptor binding domain (RBD) was relatively unaffected, yet viral titer dropped to 10% that of the parental vector (Table 1), supporting a functional role for the PRR in viral entry. Furthermore, the finding that coexpression of wild-type envelope was necessary to rescue viral infectivity despite confirmation of binding via the anti-HER2 antibody, also suggests that ZZ insertion into the PRR may result in impairment of envelope fusion.

Interestingly, AZE-ZZ-GFP showed a significantly greater impairment of titer compared to wild-type AZE-GFP than ZV-ZZ-GFP compared to ZV-GFP. Previous work by Lavillette et al. (Lavillette *et al.*, 2001b) suggests that activation of fusion requires optimal interaction between the RBD and the C-terminal domains of SU. Receptor binding-activated fusion of chimeric ampho/eco envelopes similar to AZE-ZZ-GFP was reported to be less readily achieved compared to wild-type envelope because of low compatibility between these domains, requiring more time and/or more envelope-receptor interactions to occur, and this may have contributed to the greater impairment in titer. It is also notable that, although ZV-ZZ-GFP showed equivalent titers in the presence or absence of antibody, the titer of AZE-ZZ-GFP in the presence of antibody was reduced 10-fold compared to AZE-ZZ-GFP without antibody. It is possible that, in addition to effects due to the insertion of the Z domain per se, the chimeric AZE-ZZ-GFP envelope may also be more sensitive than ZV-ZZ-GFP to steric hindrance effects due to complex formation with antibodies, resulting in reduced titers in this situation.

With coexpression of the wild-type envelope, antibody-mediated enhancement of infectivity on the order of 10-fold was achieved on NIH/189 murine target cells. These results are comparable to those reported by Morizono *et al.* (2001), who used a Z domain-modified Sindbis envelope to pseudotype MLV and HIV-based vectors, and found enhancements of 10-fold (transduction levels: 2.8% untargeted vs. 28.2% targeted) and 28-fold (1.6% untargeted vs. 45.6% targeted), respectively, using an anti-HLA antibody. However, it was notable and intriguing that, although targeted virions could bind to both NIH/189 and SK-BR-3 via a specific binding interaction that could be competed by excess anti-HER2 antibodies, enhanced infectivity could be demonstrated only on NIH/189 cells. This was presumably due to subtle differences in cellular parameters that affect postbinding entry events when viral tropism is retargeted. While the principle of using targeting moieties to redirect retrovirus tropism is now well established, the low efficiency of targeting observed in most studies has generally been attributed to the lack of efficient coupling between redirected binding and virus-cell membrane fusion events in targeted vectors (Zhao *et al.*, 1999; Weber *et al.*, 2001). However, potentially critical parameters such as the type of receptor targeted and its level of cell surface expression, cell surface localization, postbinding internalization and intracellular fate, etc., have yet to be rigorously investigated. To our knowledge, this is the first direct demonstration that cellular parameters may significantly affect vector targeting efficiency and that results obtained from artificially engineered target cell lines may be different from those obtained with natural target cells expressing the same surface antigen, even despite complementation with fully fusion-competent wild-type envelope.

It has previously been observed that even nonviral protein-DNA conjugates targeted to HER2 show significant differences in transduction efficiency between NIH 3T3 cells engineered to express the HER2 target molecule at levels comparable to the SK-BR-3 human breast cancer cells, with the efficiency of HER2-directed gene transfer into the SK-BR-3 cell line only 1.5% of that seen in NIH 3T3 cells expressing HER2 (Foster and Kern, 1997). If receptor density is comparable, cell type-specific differences in the regulation or interactions of targeted receptor proteins with other cellular constituents might also affect the efficiency of ligand-mediated virus entry. In the case of HER2, differences in associated cellular constituents such as integrins (Falcioni *et al.*, 1997), ERBIN (Borg *et al.*, 2000), CD44 (Carraway and Sweeney, 2001), or PICK1 (Jaulin-Bastard *et al.*, 2001), might account for some of the discrepancies observed when artificial target cell lines are used instead of natural target cells. For example, physical interactions between HER2 and $\alpha 6\beta 4$ integrins have been reported in human epithelial carcinoma cells, while fibroblasts such as NIH 3T3 have high levels of $\alpha 6$ but no detectable $\beta 4$; indeed, without concomitant transduction of $\beta 4$, overexpression of HER2 alone is

not sufficient to confer proliferative and invasive functional properties to NIH 3T3 fibroblasts (Falcioni *et al.*, 1997). In the absence of associated $\alpha 6\beta 4$ integrins, overexpressed HER2 molecules on the surface of the NIH/189 target cells may associate more freely with PiT-2 and allow binding via anti-HER2 antibodies to be more efficiently followed by entry via wild-type amphotropic envelope-mediated fusion, whereas the physical interactions between HER2 and $\alpha 6\beta 4$ integrins might function to inhibit HER2-targeted virus entry in human breast cancer cells.

Our results indicated that the 10.0-kb ZE-ZZ-GFP is much less stable than the 9.6-kb ZE-GFP, thus accounting for the sudden change in replication kinetics observed by FACS after the first infection cycle. In an earlier study of MLV RCR vectors, virions with 9.6-kb or smaller genomes could be produced and stably propagated over multiple serial infection cycles, but virions with 9.9-kb genomes were found to be unstable (Logg *et al.*, 2001a). Additionally, it has been shown that the replication efficiency of retroviruses has been reported to decrease with increasing virus size (Gelinas and Temin, 1986), perhaps because of the relatively low processivity of reverse transcriptase as well as packaging size limitations. Full-length ZE-ZZ-GFP replicates with significantly attenuated kinetics and thus may be rapidly overgrown by the shorter replication-competent revertants after deletion of the Z domain. These results also suggest that reducing the total size of the RCR vector genome by use of the shorter VEGF IRES may have partially achieved the intended effect to stabilize the Z domain sequence in the envelope. However, this construct was still not entirely stable, and it is likely that the presence of two identical Z domain repeat sequences in tandem may have contributed to its instability. Significantly, even though the IRES-GFP sequence also contributes to the overall size of the genome and yet is nonessential for viral replication, deletions were selective for the Z domain first, and the IRES-GFP sequence remains stable for many passages (data not shown). This preferential deletion of the Z domain sequence suggests that it is in fact recombinogenic, and may also impair viral infectivity specifically because of its position in the PRR of the envelope as well as its contribution to the overall size of the genome.

Interestingly, Kayman and co-workers (1999) have previously reported that the hypervariable domain of the PRR does not contain any specific sequence that is essential for envelope function and can tolerate deletions of up to half of its C-terminal sequence; furthermore, they reported that the C-terminal region was permissive for insertion of an entire anti-HIV single-chain antibody sequence, which was stably retained. However, these experiments were performed using the Friend MLV envelope, whose permissiveness for insertions may differ somewhat from that of the longer amphotropic MLV envelope PRR, and examination of replication kinetics was performed for only a single infection cycle. Furthermore, in spite of the demonstration that the single-chain antibody sequence was incorporated into envelopes and could be displayed in a conformation that appeared to be functionally competent for binding to its cognate epitope on an artificial target cell line, specific infectivity mediated through antibody-targeted binding was not demonstrated. In contrast, the ability to capture preexisting antibodies by use of the ZZ domain allows a more flexible and modular method for targeting that does not require construction and testing of a newly engineered envelope for each antibody. Furthermore, single chain antibodies do not always retain the same level of antigen binding affinity as the mAb from which they are derived, and it has previously been demonstrated that different single chain antibody sequences incorporated into the MLV envelope may affect the structure, assembly, and function of the protein in different ways (Benedict *et al.*, 1997, 1999).

The use of RCR vectors for cancer gene therapy allows highly efficient gene delivery from a small initial inoculum because of amplification inherent in the replicative process. Further safeguards that will minimize the risk to normal cells include targeting the retrovirus specifically and exclusively to tumor cells in order to limit and control the replicative process, and incorporation of suicide genes in the vectors to serve as a "self-destruct" mechanism. Here we have demonstrated that it is possible to achieve enhanced selectivity for cancer cells by physical targeting of RCR vectors through modification of viral tropism; antibody-mediated targeting thus represents a modular strategy that would primarily be useful for initial localization of RCR vectors to tumors. After this, retention of the Z domain is not necessary and might even be undesirable as this might result in capture of nonspecific serum antibodies after the initial infection cycle. Subsequent intratumoral spread of replicative vectors would then result in amplification of the initial low efficiency but target cell-specific infection, and further local spread could be controlled through transcriptional regulation (Logg *et al.*, 2002).

ACKNOWLEDGMENTS

We thank Dr. Frederick Hall, University of Southern California, for kindly providing plasmid pZ/Cee-S. C.-K.T. is the recipient of a Susan G. Komen Breast Cancer Foundation dissertation research award and fellowship, and C.R.L. is the recipient of a Breast Cancer Research Project Fellowship through the Norris Comprehensive Cancer Center. This work was supported by a pilot project grant through the USC/Norris Breast Cancer Research Program, a grant from the National Cancer Institute (#CA48780), and Department of Defense Breast Cancer Research Program grant #DAMD17-99-1-9377.

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Received for publication October 8, 2002; accepted after revision April 16, 2003.

Published online: May 6, 2003.